MAPPING GENES INVOLVED IN FREEZING TOLERANCE IN A BACKCROSS OF *CITRUS* AND *PONCIRUS* USING A LINKAGE MAP OF ISOZYMES AND RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

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MAPPING GENES INVOLVED IN FREEZING TOLERANCE IN A BACKCROSS OF *CITRUS* AND *PONCIRUS* USING A LINKAGE MAP OF ISOZYMES AND RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

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Improving citrus cold hardiness has been a major objective of citrus breeders since 1897; yet no cultivars with improved hardiness have been released. The genetic mechanisms responsible for cold hardiness remain poorly understood. The purpose of this study was to map genes influencing citrus cold hardiness using a linkage map of isozyme and restriction fragment length polymorphism (RFLP) markers.

Cold-acclimation-induced changes in freezing tolerance and translatable RNA content were compared in seedlings of a relatively cold sensitive citrus species (*Citrus grandis* (L.) Osb.) and a cold-hardy citrus relative (*Poncirus trifoliata* (L.) Raf.). *C. grandis* and *P. trifoliata* were able to cold acclimate and tolerate freezing to -8.5 and -18.3°C, respectively, under controlled, laboratory conditions. Qualitative changes in the *in vitro* translation profile, revealed by two-dimensional

gel electrophoresis, were observed following cold acclimation in both species, but were more pronounced for *P. trifoliata*.

Using *C. grandis* as the recurrent parent and *P. trifoliata* as the donor parent, an intergeneric backcross population was created for the purpose of constructing a genetic linkage map comprised of isozyme and RFLP markers. Sixty-five progeny were analyzed for a total of 57 markers including nine isozymes and 48 RFLPs. Linkage analysis revealed that 52 markers mapped to 11 linkage groups while five markers remained unmapped. The total map distance of the 11 linkage groups was 553 cM.

Leaves of 58 of the 65 backcross progeny used in the mapping study were subjected to controlled freezes at -8, -10, -12, and -14°C. Relative freezing tolerance of the genotypes was measured by electrolyte leakage and the temperature at which 50% of leaf tissue was damaged (the LT_{50} value) was estimated for each genotype. A comparison of the average LT_{50} value of progeny grouped according to their genotypes at marker loci revealed that at least seven quantitative trait loci (QTLs) were responsible for freezing tolerance in the backcross population. Four QTLs were detected that positively influenced freezing tolerance while three QTLs were detected that negatively influenced freezing tolerance.

CHAPTER 1
INTRODUCTION

Citrus is classified as a cold-sensitive evergreen of tropical and subtropical origin, yet some of the most valued citrus crops are grown in relatively high-risk freeze areas throughout the world (Yelenosky, 1985). The susceptibility of the Florida citrus industry to freezes was demonstrated several times in the 1980s. In the first half of that decade, an unprecedented series of four severe freezes caused crop losses in excess of $2.5 billion and led to a reduction in production acreage of nearly 25% (Parsons et al., 1986). As a result of these freezes, much of the industry has relocated to the southern portion of the state. In the traditional citrus production region of Central Florida, where replanting has occurred, the cultivars planted were no better able to withstand freezes, from a genetic perspective, than their predecessors. Despite nearly 100 years of active and vigorous breeding efforts in citrus cold hardiness, no commercially important cultivars with improved cold hardiness have been released (Cooper et al., 1962, Barrett, 1981; 1985).

The application of molecular biology techniques to plant science has the potential to enhance the efficiency of plant breeding. One tool that may become particularly useful to plant breeders is a new type of genetic marker, restriction fragment length polymorphisms (RFLPs). Tanksley et al. (1989) list four applications of RFLPs to plant breeding: 1) to expedite the movement of

desirable genes among cultivars, 2) to allow the transfer of novel genes from related wild species, 3) to dissect complex polygenic characters into Mendelian factors, and 4) to compare genetic relationships between sexually incompatible species. A prerequisite for all of these applications is the creation of a genetic linkage map in the species being studied. Such maps are becoming available in an ever increasing number of species where they are being used to identify and manipulate genes of agricultural and economic importance (Helentjaris and Burr, 1989). Linkage maps have the potential to enhance cultivar improvement and genetic analyses in perennial fruit crops, including citrus; however, there have been no reports of linkage map construction for any perennial fruit species.

The goal of this study was to create a genetic linkage map in citrus and to demonstrate a potential application of this map by using it to identify genes that may influence citrus cold hardiness. This was accomplished by constructing an intergeneric backcross between *Citrus grandis* (L.) Osbeck and *Poncirus trifoliata* (L.) Raf. using the former as the recurrent parent. The research involved in this study is discussed in the following chapters. A review of the literature is presented in Chapter 2. In Chapter 3, the changes in freezing tolerance and translatable RNA content resulting from cold acclimation of *C. grandis* and *P. trifoliata* were compared. It was reasoned that if changes were evident in translation profiles between cold-acclimated and nonacclimated *P. trifoliata*, a cDNA library constructed from RNA isolated from cold-acclimated tissue might be a useful source of clones for RFLP analysis and in mapping quantitative traits involved in freezing tolerance. In Chapter 4, the creation of the genetic linkage map is discussed, followed in Chapter 5 by the demonstration that genes localized

to certain regions of the map may act to influence citrus freezing tolerance. An overall summary of the research, as well as a discussion of other potential uses of the linkage map, are included in Chapter 6.

CHAPTER 2
LITERATURE REVIEW

Introduction

The purpose of this chapter is to provide the reader with information that will aid in understanding the research discussed in this dissertation. The first section deals with citrus breeding and addresses some of the obstacles encountered by scientists in this area of research. Next is a discussion of genetic linkage maps in plants. Specifically, the use of isozymes and restriction fragment length polymorphisms as markers in constructing such maps is discussed. Examples of how such maps have been used to identify and manipulate economically important traits are included. The chapter concludes with a section on citrus cold hardiness.

Citrus Breeding and Genetics

Active breeding programs for citrus have existed in Florida since 1892 (Cooper et al., 1962), yet most of the cultivars in current production have arisen, not through controlled pollination and selection, but as chance seedlings or as the result of natural mutations that have given rise to bud sports (Hodgson, 1967). The result is that the citrus industry of the United States, and those of most other countries, are based on only a few, closely related, cultivars (Hearn, 1985). The lack of success at breeding citrus can be attributed to a combination of the

general barriers which impede genetic improvement of fruit trees and the specific reproductive biology of the genus.

In general, successful fruit breeding involves the mating of genotypes having desirable qualities, growing large numbers of progeny from these crosses, and selecting a few progeny that have the combined desirable characteristics of their progenitors. These selected progeny are then extensively evaluated and a few may eventually become named cultivars useful to the industry and serve as parents, along with unnamed selections, in producing new generations of progeny from which further selections are made. In plant breeding terms, such a process is termed recurrent mass selection (Bringhurst, 1983). The efficiency of mass selection is influenced by several factors: 1) the frequency at which desirable traits of the parents are passed on to their offspring (the heritability of the trait); 2) the number of progeny one is able to evaluate; and 3) the amount of time necessary to carry out one generation of selection (Hansche, 1983). In tree fruit breeding, large plant size and long juvenility periods limit the number of progeny that can be evaluated because of the large amounts of resources and time needed to grow the progeny to fruiting. Fortunately, however, many traits of economic importance in fruit crops have relatively high heritabilities, although this does little to offset the constraints imposed by tree size and juvenility (Hansche, 1983).

Citrus breeding is exacerbated by several factors. Most citrus cultivars appear to be highly heterozygous (Cameron and Frost, 1968), thus the progeny produced by controlled hybridization rarely closely resemble either parent (Hearn, 1973). This necessitates the screening of large numbers of progeny before one is found that combines the desirable characteristics of its parents. Frequently, crosses

between closely related parents result in progeny with reduced vigor (Barrett, 1981; Cameron and Frost, 1968). Certain cultivars exhibit pollen sterility and self-incompatibility, limiting their use in crosses (Hodgson, 1967). Also, few traits in citrus appear to be under simple genetic control (Vardi and Spiegel-Roy, 1978). Finally, long juvenility periods are characteristic of citrus seedlings and their immediate budded progeny (Soost and Cameron, 1975). First flowering in seedlings may require five or more years and juvenile traits such as thorniness, upright growth habit, alternate bearing, and differences in fruit characteristics, may persist for even longer (Cameron and Frost, 1968; Soost and Cameron, 1975). Potential cultivars must be evaluated for several years before being released, as evidenced by the lapse of 18 years from the time of first seed germination to the release of 'Sunburst' citrus hybrid by the U.S.D.A. (Hearn, 1979).

Efficient breeding in citrus, however, is most inhibited by its reproductive biology. The seeds of most citrus species, including those of economic importance, are polyembryonic with most of the embryos being derived from the nucellus (Frost and Soost, 1968). The zygotic embryo, if present, often succumbs to competition with the nucellar embryos for nutrients during development. The result is that the majority of seedlings arising from polyembryonic seeds are identical to the maternal parent, which prevents certain otherwise superior cultivars from being used as maternal parents in a breeding program. Since polyembryony is dominant to monoembryony and likely controlled by more than one gene (Cameron and Soost, 1979; Parlevliet and Cameron, 1959), it is also undesirable to use these genotypes as pollen parents since their progeny may in

turn be polyembryonic. Most success to date in breeding citrus has been achieved with the use of maternal genotypes that produce a high frequency of zygotic embryos (Hearn, 1985).

Several ways of overcoming the problems imposed on citrus breeding by nucellar polyembryony have been suggested. These include methods that alleviate the need for sexual reproduction such as screening existing cultivars for desirable mutations, either naturally occurring mutations (Hensz, 1981), those induced by radiation treatments (Hearn, 1986), or mutants selected by in vitro methods (Ben-Hayyim and Goffen, 1989). However, these techniques do little to increase the genetic diversity of citrus cultivars and thus may predispose the industry to outbreaks of pests or unforseen enviornmental stresses (Barrett, 1977; Hearn, 1973). Others have suggested somatic hybridization (Grosser and Gmitter, 1990; Vardi and Galun, 1988) as a means to overcome nucellar polyembryony. Somatic hybridization, however, results in polyploids which can be less vigorous and less fruitful than diploids (Soost and Cameron, 1975). The greatest potential for somatic hybrids is probably in the development of new rootstocks. Also the use of biochemical markers, including isozymes, to identify zygotic progeny from crosses involving polyembryonic maternal parents has been suggested (Iglesias et al., 1974; Ortiz et al., 1981; Torres et al., 1978; Vardi and Spiegel-Roy, 1978). Although there is great potential for this approach, the actual numbers of zygotic progeny produced may remain low due to apomixis.

Overall, a better understanding of the genetic mechanisms underlying economically important traits in citrus is desirable. The best way to accomplish this may be through the construction and use of a genetic linkage map.

Genetic Linkage Maps in Plants

Well defined linkage maps have been constructed for several plant species, especially maize (*Zea mays* L.), tomato (*Lycopersicon esculentum* Mill), and *Arabidopsis thaliana* L. Classical linkage maps in maize and tomato, based on morphological characters, have existed since the early 1900s. The advent of isozyme technologies in the late 1950s and the development of techniques for the analysis of variability at the DNA level in the mid to late 1970s greatly expanded the types of genetic markers available for linkage maps in these species. Furthermore, the construction of linkage maps is now theoretically possible for any plant species.

History of Biochemical and Molecular Genetic Markers

Isozymes. Isozymes are the most common form of biochemical genetic markers. The late 1950s saw the advent of this new type of genetic marker made possible by combining the techniques of starch gel electrophoresis (Smithies, 1955) and enzyme activity staining (Hunter and Markert, 1957). Isozymes are defined as different molecular forms of an enzyme that catalyze the same reaction. Although several mechanisms have been suggested that give rise to isozymes (Weeden, 1983), plant breeders and geneticists are generally only concerned with allozyme variation, i.e., allelic differences at a structural locus for an enzyme (Prakash et al., 1969). The more common term isozyme will be used throughout this dissertation.

Peirce and Brewbaker (1973) were the first to suggest the potential use of isozymes in horticultural science. Much of the early work, however, only included descriptions of polymorphisms in isozyme banding patterns observed among

various genotypes. Later, genetic analyses of isozyme loci became more common. When Torres (1983) reviewed the literature there were only a few cases of isozyme analysis in fruit crops. Most notably, of the major fruit crops, genetic analysis of isozyme loci had been performed in citrus and avocado (*Persea americana* Mill.). In more recent reviews, the crops for which isozymes have been genetically analyzed include most of the major fruit species (Torres, 1989; Weeden, 1989); however, too few isozyme markers are known in these species to permit their use alone in genetic mapping studies.

Restriction fragment length polymorphisms. Restriction fragment length polymorphisms (RFLPs) are now the most common type of molecular genetic marker. RFLPs are defined as homologous fragments of DNA that vary in molecular weight (length) following cleavage with DNA restriction endonucleases, enzymes that cleave DNA at specific base pair sequences. Such polymorphisms are due to either base pair changes at the recognition site, to a rearrangement encompassing that site, or to insertion/deletions events within the DNA fragment (Helentjaris, 1987). The use of RFLPs as genetic markers was first proposed in humans (Botstein et al., 1980) and the first genetic map of man, based on RFLPs was reported by Donis-Keller et al. (1987). In 1983, reports began to appear in the literature indicating the potential applications of RFLPs to plant breeding (Burr et al., 1983; Soller and Beckmann, 1983; Tanksley, 1983b) along with an estimate of the monetary resources needed for such analyses when used in genetic improvement programs (Beckmann and Soller, 1983). Additional reviews of the potential uses of RFLPs have been published (Helentjaris et al., 1985; Beckmann and Soller, 1986; Landry and Michelmore, 1987) and Tanksley et al. (1989) gave

several examples of how RFLPs have been used in plant breeding. The first linkage maps in plants based on RFLP analysis were reported for maize and tomato (Helentjaris et al., 1986; Bernatzky and Tanksley, 1986b). Partial genetic linkage maps, based on RFLPs, are now available in several additional plant species including lentel, *Lens culinaris* Medik. (Harvey and Muehlbauer, 1989), lettuce, *Lactuca sativa* L. (Landry et al., 1987), pepper, *Capsicum annuum* L. (Tanksley et al., 1988), potato, *Solanum tuberosum* L. (Bonierbale et al., 1988; Gebhart et al., 1989), and rice, *Oryza sativa* L. (McCouch et al, 1988).

Summary of the Methodology Needed for Isozyme and RFLP Analysis

Isozymes. The analysis of isozymes involves three major steps: sample preparation, electrophoresis, and enzyme activity staining. Sample preparation is usually accomplished by grinding a small amount of tissue in a buffered solution and absorbing the sample into filter paper wicks which are then inserted into a gel. Electrophoresis is usually performed in starch gels, although, for some applications, polyacrylamide may be used. In the case of polyacrylamide, samples are first cleared by centrifugation and then loaded directly into pre-formed wells in the gel. Electrophoresis is carried out under native (nondenaturing) conditions and protein separation is according to both size and charge. Following electrophoresis the gel is subjected to enzyme activity staining where all necessary substrates and cofactors, including some type of indicator compound, are added in solution to the gel and particular enzymes are detected by the formation of a chromatic region in the gel. The methodology involved in isozyme analysis has been reviewed in detail by Shields et al. (1983).

RFLPs. Several prerequisites must be met before analysis of RFLPs can begin. A simple method for extracting DNA from the plant being studied must be available. The method of Dellaporta (1983) has been reported to work well for a wide variety of species while more specific techniques can be found in the RFLP literature for other plants. Also, a source of cloned DNA sequences must be available for use as probes in RFLP analysis. Most probes are selected from some type of DNA library, a collection of DNA fragments representing a subset of an organism's genome with each fragment being individually propagated (cloned) in a microorganism, usually the bacterium *Escherichia coli*. This usually includes either cDNA libraries, i.e., DNA fragments that are synthesized from mRNA thus representing the transcribed portion of an organism's genome (Bernatzky and Tanksley, 1986a), or genomic DNA libraries enriched for unique or low copy number sequences (Burr et al., 1988; Landry and Michelmore, 1985). Heterologous probes of cloned genes from other species have also been used successfully (Vallejos et al., 1986).

Blotting of DNA for RFLP analysis is performed basically as described by Southern (1975). First, DNA is isolated from tissue, usually leaves, and is cleaved with a restriction enzyme. The DNA is then electrophoresed in an agarose gel, usually 0.8% to 1.2% agarose, denatured (i.e., made single stranded) by soaking the gel in 0.5 *M* NaOH, and transferred by capillary blotting to a solid support matrix of nitrocellulose. The DNA is then irreversibly bound to the matrix by vacuum drying under high heat (80°C). Prior to hybridizing a radioactively labeled probe to the DNA blot, the matrix is first prehybridized to block all other DNA binding sites, thus avoiding nonspecific binding of the probe to the

nitrocellulose. The blot is then hybridized to the labeled probe for several hours after which excess label is removed by washing the blot in solutions containing decreasing concentrations of salt. X-ray film is then exposed to the blot from one to several days with intensifying screens. Following autoradiography, the probe can be removed from the blot by denaturing it from the bound DNA using either high heat or alkaline conditions and the blot can be reused. In applications of DNA blotting where it is desired to reuse the blot several times, such as RFLP analysis, the use of nylon membranes has been found to be superior to the more conventionally used nitrocellulose. In this case, the blotted DNA is crosslinked to the membrane using UV light and different conditions for prehybridization and hybridization are used (Church and Gilbert, 1984).

Useful Characteristics of Molecular Markers

There are several reasons why molecular markers are advantageous over conventional, morphological genetic markers (Tanksley, 1983a, 1983b; Tanksley and Rick, 1980; Tanksley et al., 1982; Tanksley et al., 1989). Unlike morphological markers, molecular markers exhibit phenotypic neutrality, they are inherited codominantly, they rarely exhibit epistatic or pleiotropic interactions, and they can be detected in both juvenile and adult tissue. These characteristics combine to allow a theoretically limitless number of marker loci to be analyzed in a particular segregating population thus allowing the easy construction of genetic linkage maps. The construction of a linkage map is accomplished by evaluating many markers, both isozymes and RFLPs, segregating in usually a backcross or F_2 hybrid population. The marker loci are first individually characterized to confirm Mendelian inheritance. All pairwise combinations of loci are then tested for

independent assortment; statistically significant deviations from expected ratios imply that loci are linked. Distances between linked loci are expressed in units of recombination, centiMorgans (cM), with 1 cM equal to 1% recombination when using a backcross population. Three-point, or in some cases, multipoint, analysis is used to confirm gene order in regions of a chromosome that may be densely populated with markers. Once enough markers are evaluated, the genome becomes saturated with markers, and the number of linkage groups elucidated will equal the n number of chromosomes characteristic of the organism.

Isozymes have the advantage over RFLPs in that their analysis can be performed more rapidly and less expensively than RFLPs. However, for many applications, the number of isozyme markers available in a particular crop is a limiting factor (Tanksley, 1983b). RFLPs, while technically more expensive and difficult to analyze, do not require specific techniques such as enzyme activity staining and appear to be more efficient (i.e, because of their abundance) than isozymes at detecting polymorphisms within segregating populations. For example, in 1983, 30 isozyme loci had been mapped in tomato (Rick, 1983) and 37 in maize (Goodman and Stuber, 1983). By 1988, over 300 additional markers, RFLPs, had been mapped in each of these crops (Helentjaris, 1987; Young et al., 1988).

General uses of Molecular Markers

The uses of isozymes in plant genetics and breeding have been well documented in several reviews (Tanksley and Orton, 1983; Nielsen, 1985; Weeden, 1989). Tanksley et al. (1989) recently reviewed work involving RFLPs and a collection of extended abstracts from a recent meeting dealing with the

application of RFLPs to plant genetics and breeding has been published (Helentjaris and Burr, 1989).

In some cases, only one or a few markers need to be analyzed and it is often unnecessary to know the location of these markers in the plant's genome. Such applications include: 1) identification of sexual and parasexual hybrids; 2) estimation of outcrossing rates; and 3) inferring taxonomic relationships among related species and genera. Since only a few molecular markers are necessary for such applications, isozymes have been and likely will continue to be most used for these applications. Examples specific to citrus of these applications can be found in the literature.

The work of Torres and associates has identified nine enzyme systems in citrus encoded by 12 polymorphic loci (Soost and Torres, 1981; Torres et al., 1978; 1982; 1985). Linkage analysis among these loci revealed two linkage groups, one comprised of *Got1* and *Mdh1*, and the other comprised of *Me01*, *Me02*, and *Mdh2* (Torres et al., 1985).

The use of isozymes to identify sexual and parasexual hybrids has been of particular benefit in citrus breeding. As mentioned earlier, their use in distinguishing zygotic from nucellar seedlings should allow breeders to discard nucellar seedlings from breeding populations at a very early stage (Iglesias et al., 1974; Torres et al., 1978). This was demonstrated by Soost et al. (1980) who, on the basis of isozyme analysis, discarded 18 nucellar progeny from a population of 123 seedlings in a cross of 'King' tangor X 'Parsons Special' mandarin. The production of a high frequency of nucellar seedlings is desired in rootstock

cultivars of citrus because clonal propagation of the genotype is thus readily achieved. Isozyme analysis has allowed researchers to estimate the frequency of zygotic seedlings produced by various rootstock cultivars and analysis of bark isozymes even allows for rootstock evaluation of long established plantings (Ashari et al., 1988; Hirai et al., 1986; Khan and Roose, 1988; Moore and Castle, 1988; Roose and Traugh, 1988). Isozymes have also been used to identify products of protoplast fusion (Ben-Hayyim et al., 1982; Grosser and Gmitter, 1990).

Studies of phylogenetic relationships among *Citrus* species have also utilized isozymes (Button et al., 1976; Esen and Soost, 1976; Handa et al., 1986; Hirai et al., 1986). Conclusions reached by these researchers generally agree with previous taxonomic studies in citrus that suggest that there are three basic affinity groups in Citrus, pummelos (*C. grandis* [L.] Osbeck), citrons (*C. medica* L.), and mandarins (*C. reticulata* Blanco) (Barrett and Rhodes, 1976). Although much diversity in isozymes has been revealed among the various cultivated *Citrus* species and related genera, strikingly little diversity has been detected within certain citrus species. Also, within several species, including lime (*C. aurantifolia* [Christm.] Swing.), sour orange (*C. aurantium* L.), rough lemon (*C. jambhiri* Lush.), lemon (*C. limon* L.), grapefruit (*C. paradisi* Macf.), sweet orange (*C. sinensis* [L.] Osb.) and *Poncirus trifoliata* (L.) Raf., almost all cultivars exhibited the same heterozygous genotype at several loci (Torres et al., 1978; 1982). This supports the theory that most cultivars of these species may have arisen as hybrids among the three affinity groups (Barrett and Rhodes, 1976) and that additional cultivars of these species have likely arisen as bud sports or nucellar

seedlings (Hodgson, 1967). Isozyme data were used by Scora et al. (1982) to suggest sweet orange and pummelo as the progenitors of grapefruit. Sour orange has been suggested to be a hybrid between mandarin and pummelo based on isozyme analysis (Hirai and Kajiura, 1987).

Gene Mapping

The identification and manipulation of economically important traits is the prime objective of most plant breeders. While some traits may be monogenic in nature and easily evaluated, the evaluation of other traits may be difficult. The evaluation of pathogen resistance, for instance, often requires difficult procedures for inoculation and monitoring disease progression or expression may be environmentally dependent. In other cases, certain potentially destructive races of a pathogen may be unavailable due to quarantine restrictions. The manipulation of such traits is simplified if the gene or genes responsible for the trait can be "tagged" with a marker gene. Gene tagging refers to the identification of an allele at a marker locus that is associated with a particular allele of interest at another locus. The extent of the association depends on the linkage strength between loci. Selection for the trait is then accomplished by selecting for the marker allele rather than the trait itself. The classic example of such analysis is from tomato.

Rick and Fobes (1974) found a tight linkage between an isozyme locus, *Aps-1*, and a gene responsible for nematode resistance. This discovery has been extremely useful to tomato breeders since screening for nematode resistance is both difficult and time consuming, while isozyme analysis is relatively quick and easy. *Aps-1* monitoring has become widely adopted by private firms and public

agencies in breeding for nematode resistance in tomato (Rick, 1983). This discovery has encouraged other researchers to try to identify linkages of isozyme loci and genes controlling economically important traits. Several studies have met with success (for review see Weeden, 1989). However, the number of useful isozyme markers remains a limiting factor in most analyses (Tanksley, 1983b). The growing abundance of RFLPs has ameliorated this problem. Weeden (1989) stated that with the abundance of RFLPs available for some species, it is theoretically possible to map any monogenic trait in any cross. Tanksley et al. (1989) report that in tomato, genes for resistance to tobacco mosaic virus, *Fusarium* wilt, bacterial speck and root knot nematodes have all been linked to RFLPs as have genes for resistance to dwarf mosaic virus in maize and downy mildew in lettuce. With an ever growing number of markers, it is even possible to map genes involved in the expression of quantitative traits.

Mapping Quantitative Trait Loci

Many traits of economic significance are not simply inherited but rather exhibit continuous or quantitative variation. The variation for quantitative traits has been explained by the simultaneous segregation of many genes (polygenes) affecting the trait, and the interaction of these genes, both individually and corporately, with the environment (Falconer, 1981; Simmons, 1979). The chromosomal locations of individual genes involved in the expression of quantitative traits have been termed quantitative trait loci or QTLs (Geldermann, 1975). QTLs (though not referred to as such) were first demonstrated in plants. Sax (1923), working with beans (*Phaseolus vulgaris* L.), identified an association between a locus controlling seed size, a quantitative trait, and seed coat

pigmentation, a simply inherited trait. He concluded that this association was due to linkage of genetic factors affecting seed size to alleles conditioning pigmentation. Later, studies with maize, using reciprocal translocations as chromosomal markers, indicated that resistance to certain pests (both disease and insect) was conditioned by genes on several chromosomes (for review see Robertson, 1989). Other studies have also demonstrated the presence of QTLs in proximity to marker loci (Thompson and Thoday, 1979). Even though these early studies were successful at identifying chromosomal regions involving quantitative traits, little practical use has been made of the information (Robertson, 1989).

The often deleterious effects of morphological or chromosomal mutant alleles, as well as the abundance of pleiotropy and/or epistasis usually associated with these markers, limit their use in gene mapping studies. This is true of studies involving QTLs as well. In fact, it is in the latter studies where the researcher desires to have an abundance of simply inherited traits segregating in a single population. Their abundance, phenotypic neutrality and apparent lack of epistatic or pleiotropic effects make molecular markers ideal for such studies. In species where saturated linkage maps exist, markers can be chosen that are spaced evenly along each chromosome, at a distance of 10 to 20 cM, and that segregate in a population also exhibiting extreme variation for a quantitative trait or traits. The researcher then evaluates the population for the quantitative trait while also conducting genetic analysis of the marker loci. Tests for associations between the segregation of the marker loci and the quantitative trait(s) are then carried-out. Such associations, when they are detected, suggest that the marker locus is linked to a locus involved in the expression of the quantitative trait.

There are essentially two approaches in mapping QTLs (Lander and Botstein, 1989). The first, or traditional, approach involves statistically comparing the phenotypic means of the segregating progeny grouped according to their genotype at a particular marker locus. This analysis provides an estimate of the phenotypic effect of an allele substitution, i.e., AA vs. AB vs. BB for an F_2 population or AA vs. AB for a backcross, and, in the F_2 population, allows the researcher to distinguish the mode of action of the QTL as either additive or non-additive (a linear effect of allele substitution suggests additive gene action). While the traditional approach encompasses the key features of QTL mapping, it has several shortcomings (Lander and Botstein, 1989). Chiefly, the location of the QTL in reference to the marker locus is unknown, thus one cannot distinguish between tight linkage of a QTL with small effects and loose linkage to a QTL with large effects. Nevertheless, the mapping of several QTLs has been demonstrated using this technique. Many of the initial QTL studies involved isozymes as genetic markers (reviewed in Weeden, 1989); however, recent analyses have also utilized RFLPs (Helentjaris, 1987; Martin et al., 1989; Nienhuis et al., 1987; Osborn et al., 1987; and Tanksley and Hewitt, 1988). The use of molecular markers for the study of QTLs in maize has been recently reviewed by Stuber (1989).

The second approach involves a technique known as interval mapping (Lander and Botstein, 1989) and is employed when genetic markers have been scored throughout a genome. The traditional approach of analyzing each locus independently may not detect a QTL with statistical significance due to recombination of the QTL and the marker locus. However, the approach of

interval mapping ameliorates this problem by basing analyses on two linked markers bounding an interval which may contain a QTL (Tanksley et al., 1989). The method of maximum likelihood is used to calculate LOD (log of the odds) scores that indicate the probability of the data having arisen assuming the presence of a QTL versus no QTL being present. A plot of the LOD scores along a chromosomal segment is indicative of where on a chromosome a QTL may be located (Lander and Botstein, 1989). Paterson et al. (1988), using a saturated linkage map of RFLPs and isozymes in tomato, were able to detect several QTLs affecting quantitative traits. They investigated three quantitative traits (fruit weight, soluble solids content, and fruit pH) segregating in a backcross of a wild tomato species from Peru, *Lycopersicon chmielewskii*, to the cultivated tomato, *L. esculentum*. They distinguished at least six QTLs controlling fruit weight, four QTLs for soluble solids content, and five QTLs for fruit pH, which correlated with 58%, 44%, and 48%, respectively, of the phenotypic variance among the backcross progeny. Paterson et al. (1990) were further able to localize certain QTLs to chromosomal regions as small as 3 cM using a technique they called substitution mapping which involves the selection and comparison of segregating progeny having overlapping chromosomal segments. A long term goal of this project is to engineer an agriculturally-acceptable tomato with a higher yield of soluble solids (Paterson et al., 1988).

No analyses of QTLs have been conducted in any fruit crop. However, it is in perennial fruit species where such analysis may be most rewarding. Hansche (1983) indicates that a great deal of genetic potential exists for tree fruit improvement but that the rate of improvement is hindered by tree size and

seedling juvenility. The mapping and tagging of QTLs with biochemical and molecular markers offers some hope in this regard. The elucidation of linkage between marker loci and QTLs would allow for more efficient selection of quantitative traits. Progeny could be screened at the seedling stage for particular alleles at marker loci that are linked to QTLs. Only the most promising progeny would need to be grown to maturity. Thus the breeder would then have a potentially more favorable pool of progeny from which to make selections.

Breeding Citrus for Cold Hardiness

General Aspects of Cold Hardiness Genetics

Most studies involving the genetics of cold hardiness have focused on cultivar and varietal trials and the selection of cold-tolerant lines, with less attention being given to the genetic mechanisms controlling the trait (Guy, 1990; Stushnoff, 1972). However, progeny produced from parents differing in cold tolerance usually exhibit a continuous range of hardiness between the parental extremes implying that cold hardiness is a quantitatively inherited trait (Guy, 1990). While it is generally believed that many loci are involved in cold hardiness, some studies have indicated that the major genes involved may be few. Cytogenetic analysis involving the use of monosomics and substitution lines in wheat suggest that at least 10 of the 21 pairs of chromosomes are involved in freezing tolerance; however, the major genes involved in tolerance appear to be localized to chromosomes 5A and 5D (Sutka and Veisz, 1988). Similarly, Lisenfeld et al. (1986), based on segregation in F_5 lines of pea (*Pisum sativum* L.), suggested that as few as 3 or 4 linkage groups may control cold hardiness. They also found that

winter hardiness was associated with morphological markers located on chromosomes I and VI.

The role of altered gene expression during cold acclimation has also been studied. Freezing tolerance in many plants is an inducible phenomenon and increases when plants are cold-acclimated, i.e., exposed to low-nonfreezing temperatures (Levitt, 1980). Weiser (1970) proposed that cold acclimation may require the activation of genes not normally expressed during nonacclimating conditions. Changes in protein synthesis during cold acclimation have now been confirmed in many species (Guy, 1990; Singh and Laroche, 1988) including sweet orange. Cold acclimated sweet orange leaves possessed a large molecular weight polypeptide not present in nonacclimated tissue (Guy et al., 1988). Polypeptides of similar size have been reported to be induced by exposure to low temperature in other species including spinach, *Spinacia oleracea* L. (Guy and Haskell, 1987; 1988; Guy et al., 1985; 1988), and *Arabidopsis* (Gilmour et al., 1988; Kurkela et al., 1988; Lang et al., 1989). It would be interesting to determine whether such genes expressed during exposure to low temperature are located near regions exhibiting effects for cold hardiness based on QTL analysis. Should this be the case, it would be evidence that the induced protein may be directly related to the ability of the plant to survive freezing.

Citrus Cold Hardiness

Breeding citrus for cold hardiness began in 1897 after the severe freeze of 1894-95 destroyed most of the progeny produced by Webber and Swingle in their initial breeding program (Cooper et al., 1962). Through the years, the genetic sources for cold resistance have been the very cold-hardy trifoliate orange

(*Poncirus trifoliata* [L.] Raf.), kumquat (*Fortunella margarita* [Lour.] Swing.), and *Eremocitrus glauca* (Lindl.) Swing. and the somewhat less cold-hardy mandarin (*C. reticulata*) (Barrett, 1981, Soost and Cameron, 1975). Of commercially important citrus species, the mandarins are most cold hardy, followed by sweet orange and grapefruit, with lemons and limes being the least cold hardy (Yelenosky, 1985). Improvement of cold hardiness continues to be an important objective in citrus breeding (Hearn, 1985; Soost and Cameron, 1975).

That cold hardiness is heritable in crosses of citrus and its relatives was first demonstrated by Webber and Swingle (Cooper et al., 1962). In 1897 they used trifoliate orange as the cold-hardy parent in crosses with sweet orange in an attempt to develop dessert quality fruit with the characteristics of sweet orange and the cold hardiness of trifoliate orange. The hybrids produced were cold hardy, but their fruit was inedible due to the presence of acrid oil derived from the trifoliate parent. Additional crosses were made between trifoliate orange and mandarins, limes, lemons, grapefruit, sour orange, and kumquat (Cooper et al., 1962). Nearly 1,500 progeny from these crosses were evaluated and all possessed the objectional acrid flavor characteristics of trifoliate orange. Thus, none of these hybrids were of commercial value as scions because of their poor quality fruit. The fact that the acrid flavor and cold-hardy characteristics of trifoliate orange appeared in practically all hybrids led Cooper et al. (1962) to conclude that the genes that control these two characteristics are possibly closely linked on the same chromosome and the chance of breaking this linkage by subsequent crosses is not promising, but not impossible. However, several of these hybrids

have become important rootstocks such as the 'Swingle' citrumelo (*C. paradisi* X *P. trifoliata*) (Hutchison, 1974).

The early work of Webber and Swingle has continued into the present with similar crosses made by Barrett (1981). Like the prior work, Barrett's objectives were to develop cold-hardy citrus cultivars with edible fruit through intergeneric hybridization of commercially important citrus species with cold hardy citrus relatives. Barrett (1981) again demonstrated that cold hardiness in citrus is heritable with progeny of crosses between cold hardy and less hardy parents being intermediate between their parents for cold hardiness. He also found no edible fruit among F_1 hybrids of citrus and either trifoliate orange or *E. glauca*. However, first generation backcrosses of certain F_1 progeny to sweet orange, (*C. paradisi* X *P. trifoliata*) X *C. sinensis*, produced selections that closely resembled sweet orange and had edible, dessert-type fruit. Therefore, it seems that it may be possible to break the proposed linkage (Cooper et al., 1962) between acrid fruit flavor and cold hardiness. However, no cultivars with improved cold hardiness have yet been released from the breeding program outlined by Barrett (1981, 1985).

Screening Citrus for Cold Hardiness

In many instances, natural freezes have been used to discern the relative cold hardiness of various citrus genotypes (Barrett,1981; Yelenosky et al., 1968) and resistance to cold under field conditions is of utmost importance. However, the breeder's job can be performed more efficiently when cold hardiness can be evaluated under controlled conditions (Stushnoff, 1972; Young and Hearn, 1972). Young and Hearn (1972) gave three requirements for cold hardiness screening in

citrus. First, the plants must be adequately hardened. Citrus species and cultivars differ very little in their ability to withstand freezing while in a state of active growth (Yelenosky, 1985), but cold hardiness increases as a result of environmentally induced quiescence resulting from either decreased temperature or during periods of drought (Yelenosky, 1978b; Young, 1970; Young and Peynado, 1962). Therefore, it is necessary to expose citrus to low, nonfreezing temperatures, and/or reduce watering for several weeks prior to controlled freezes. Second, the procedure used to determine cold hardiness should be reproducible. Several methods have been used including freezing entire plants (Yelenosky, 1978a; 1990; Young, 1961, 1966, 1969) and freezing detached leaves (Hutcheson and Wiltbank, 1970; Wiltbank and Oswalt, 1983; 1984; Young, 1966; Young and Peynado, 1967). Methods for evaluating freeze damage include visual observations and scoring of plants following their return to an environment that promotes regrowth (Yelenosky, 1978; 1990; Young, 1961; 1966; 1969). The tolerance of tissue to internal ice has often been measured by electrolyte leakage (Wiltbank and Oswalt, 1983; 1984; Yelenosky, 1990). Third, the cold hardiness screening procedure should accommodate large numbers of plants over a relatively short period of time. In this respect, budded citrus seedlings, rather than mature trees, could be used for initial screening for cold hardiness since there is good correlation between the hardiness exhibited at the immature and mature stage (Young and Hearn, 1972).

Conclusion

This chapter has described some of the barriers encountered by citrus breeders as they attempt to understand and genetically manipulate traits of economic importance to the citrus industry. Breeders and geneticists of other crops have benefited from the use of genetic maps to increase the efficiency of cultivar improvement programs. In the following chapters, research is described that has resulted in the construction of a partial genetic map in citrus. The potential use of such a map is demonstrated by identifying certain regions of the citrus genome that may influence cold hardiness.

CHAPTER 3
COLD-ACCLIMATION-INDUCED CHANGES IN FREEZING TOLERANCE AND TRANSLATABLE RNA CONTENT IN YOUNG SEEDLINGS OF *CITRUS* AND *PONCIRUS*

Introduction

Many plants exhibit an increase in freezing tolerance when exposed to low-nonfreezing temperatures (Levitt, 1980). This process, termed cold acclimation, involves numerous physiological changes including well-documented changes in gene expression and protein synthesis (Singh and Laroche, 1988; Guy, 1990). *Citrus* and its relatives are known to cold acclimate (Yelenosky, 1985) and leaves of sweet orange (*Citrus sinensis* (L.) Osb. cv. Valencia) exposed to 5°C for 1 week exhibited altered polypeptide patterns from those maintained at 25°C (Guy et al., 1988). Most studies to date have used either seedling trees more than one year old or, alternatively, mature, clonally propagated material. The purpose of this research was to compare cold-acclimation-induced changes in freezing tolerance and translatable RNA content in very young seedlings of a relatively cold-sensitive citrus species and a cold-tolerant citrus relative.

Materials and Methods

Seed Germination and Plant Growth

Seeds of 'Pomeroy' trifoliate orange, *Poncirus trifoliata* (L.) Raf., were obtained from Willits and Newcomb, Inc., Arwin, California and seeds of 'Thong

Dee' pummelo, *Citrus grandis* (L.) Osb., were harvested from the Florida Citrus Arboretum, Division of Plant Industry, Winter Haven, Florida. In both cases seeds resulted from open pollinations. Prior to germination, seeds were stored at 4°C. Germination was accomplished in plastic seedling flats (27.3 X 54.0 X 6.4 cm) containing artificial soil medium (Metromix 300, R. W. Grace) in a 25°C growth chamber, 12 hr photoperiod. The average irradiance at plant height was 470 $\mu mol/m^2/sec$ as previously described (Guy et al., 1987). Seedling emergence was evident after 3 weeks for trifoliate orange and 1 week for pummelo. Pummelo seedlings were generally more vigorous than those of trifoliate orange.

Cold Acclimation

P. trifloiata. Flats of seedlings six-weeks old (post emergence) were transferred to a second growth chamber, where the temperature was maintained at 5°C, and the plants were allowed to cold acclimate for seven weeks.

C. grandis. Flats of seedlings three-weeks old (of comparable size to six-week old trifoliate orange seedlings) were transferred to a growth chamber at 15°C for 10 days after which the temperature was adjusted to 10°C/5°C (day/night) and the plants were allowed to cold acclimate for four weeks. The reason for the difference in cold acclimation regimes is that *C. grandis* exhibited low-temperature-induced photobleaching after only two days when transfered to 5°C constant.

Control seedlings of both genotypes were maintained at 25°C.

Evaluation of Freezing Tolerance

Following acclimation, seedlings of both species, cold acclimated and nonacclimated, were removed from soil, wrapped in a Kimwipe (Kimberly Clark,

Roswell, Georgia) tissue, and placed inverted into a glass test tube, one plant per tube. The tissue was moistened with distilled water, and the tube capped. The tube was then placed in a polyethylene glycol controlled temperature bath equilibrated to 0°C. The temperature was then lowered to -1°C and a chip of ice was added to each tube to ensure ice nucleation and freezing of surface water. The temperature of the bath was then decreased 2°C per hour and 4 plants of each treatment were removed at 2°C intervals beginning at -3°C. Following removal from the bath, tubes were placed at 5°C to slowly thaw overnight.

The next day, plants were evaluated for freezing tolerance visually and by electrolyte leakage. The visual evaluation consisted of scoring plants for the presence and degree of watersoaking. Additionally, two plants from each treatment were replanted and placed in the greenhouse under intermittent mist (10 minute intervals during daylight hours) and visually evaluated for damage and regrowth after seven and 14 days. For evaluation by electrolyte leakage, two samples of three leaves each, were removed from each plant and placed in sample vials containing 10 ml of distilled water. A total of 6 samples were evaluated for *P. trifoliata* and 4 samples for *C. grandis*. The vials were capped and allowed to incubate at room temperature with gentle shaking for two hours. The conductivity of the solution was then determined using a CDM3 conductivity meter (Radiometer Copenhagen). Following initial conductivity measurements, the vials were frozen overnight at -80°C, thawed, and a second conductivity measurement recorded. Electrolyte leakage was calculated as the ratio of the first and second conductivity measurements. The LT_{50} value for visual observations was the temperature at which approximately 50% of the leaf tissue

turned brown after seven to 14 days under greenhouse conditions. For electrolyte leakage, the LT_{50} value was arbitrarily considered to be the temperature where electrolyte leakage equaled 0.5.

RNA Isolation, *In-Vitro* Translation and Gel Electrophoresis

Total RNA was isolated from cold-acclimated and nonacclimated plants as described by Guy et al. (1985) except that tissue was ground in liquid nitrogen prior to the addition of extraction buffer. For details of RNA extraction see Appendix A. Rabbit reticulocyte lysates (Bethesda Research Laboratories, Gaithersburg, Maryland) were programmed with total RNA (5 μg) in the presence of 10 μCi L-[^{35}S]methionine. Two-dimensional gel electrophoresis of translation products was as described by Guy and Haskell (1988) with equal quantities of trichloroacetic acid insoluble radioactivity (10^5 cpm) loaded on each gel. The isoelectric focusing dimension was carried out using a carrier ampholyte composition of 0.8% pH 4-6, 0.8% pH 5-7, and 0.4% pH 3-10, in a 3.5% acrylamide O'Farrell (1975) gel. Second dimension separation was performed in 10% acrylamide gels containing 0.1% sodium dodecyl sulfate (SDS).

Results and Discussion

When exposed to low, nonfreezing temperatures, nine-week-old seedlings of *C. grandis* and 13-week-old seedlings of *P. trifoliata* were able to cold acclimate as revealed by the increased freezing tolerance of these plants when compared to non-acclimated controls (Table 3-1). *C. grandis* demonstrated an increase in freezing tolerance from -5.8°C to -8.5°C, whereas *P. trifoliata* increased from -8.8°C to -18.3°C. This is consistent with other reports indicating that *P. trifoliata*

Table 3-1. Cold-acclimation-induced freezing tolerance in *C. grandis* and *P. trifoliata*.

Genotype	Treatment	
	Nonacclimated	Cold Acclimated
	LT_{50} (°C)[a]	
P. trifoliata		
Visual observation	-6	-17
Electrolyte leakage	-8.8 (0.9)	-18.3 (2.1)
C. grandis		
Visual observation	-6	-8
Electrolyte leakage	-5.8 (0.8)	-8.5 (0.5)

[a]LT_{50} values for visual observations were based on regrowth of two samples of each genotype at each temperature treatment. LT_{50} values for electrolyte leakage were means of six observations for *P. trifoliata*, and four observations for *C. grandis*, at each temperature treatment. Standard deviations are included in parentheses for the electrolyte leakage measurements.

is more cold hardy, when cold acclimated, than *C. grandis* (Young, 1963; Ikeda, 1982). That *P. trifoliata* is able to withstand such severe temperatures has been previously reported. Yelenosky et al. (1968) observed that field plantings of *P. trifoliata* seedlings exhibited no damage when exposed to a natural freeze with an air temperature of -16°C. However, the levels of freezing tolerance observed in the present study are lower than those typically observed for *C. grandis* and somewhat lower than those previously observed for *P. trifoliata*. The greater increase in freezing tolerance observed may have been due to the cold-acclimation regimes used. Such acclimating conditions, i.e., seven weeks at 5°C constant for *P. trifoliata*, and 10 days at 15°C followed by four weeks at 10°C/5°C (day/night) for *C. grandis*, are not experienced by plants in the field and may have resulted in greater freezing tolerance following acclimation. Also, unique to this study is the observation that very young seedlings of *P. trifoliata* (6 weeks old when cold acclimation was initiated) were able to acclimate as well and that these young seedlings which are ordinarily deciduous did not lose their leaves under the conditions described. Previous reports have examined the cold hardiness of *P. trifoliata* stems, while this report is the first to demonstrate that trifoliate orange leaves, when cold acclimated, become cold hardy as well. It is interesting to note, then, that in a plant that is normally deciduous during winter, leaves still exhibit a remarkable ability to cold acclimate. Also encouraging was the fact that LT_{50} values based on electrolyte leakage closely agree with those based on visual observations following reestablishment in soil (Table 3-1).

Comparisons of polypeptides resulting from *in-vitro* translations of total RNA isolated from cold-acclimated and nonacclimated leaf tissue revealed numerous

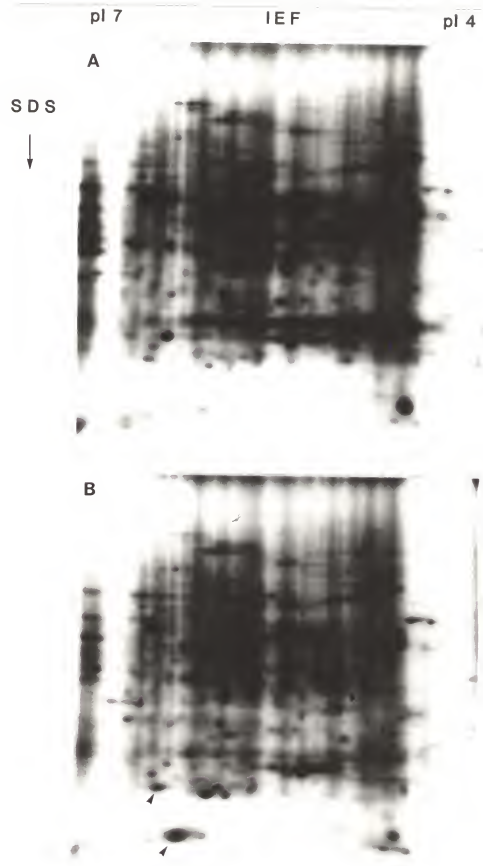
changes in *P. trifoliata* and few in *C. grandis*. The most prominent differences in polypeptide content are indicated in Figures 3-1 and 3-2. Most noteworthy was the presence of a very large molecular mass polypeptide (approximately 160 kDal) in cold-acclimated trifoliate orange that was not visible in nonacclimated leaves; nor was it detected in either nonacclimated or cold-acclimated pummelo. This is significant in that polypeptides of a similar size have been shown to be highly correlated with cold-acclimation in spinach (Guy et al., 1985; 1988; Guy and Haskell, 1987; 1988), and *Arabidopsis thaliana* (Gilmour et al., 1988: Kurkela et al., 1988; Lang et al., 1989). A similar polypeptide was also observed in cold-acclimated sweet orange (Guy et al., 1988). Whether or not the spinach, *Arabidopsis*, citrus, and *Poncirus* polypeptides share homology remains to be determined. However, the apparent lack of the 160 kDal polypeptide in cold-sensitive pummelo, which is sexually compatible with cold-hardy trifoliate orange, may eventually allow genetic analysis to demonstrate whether or not cold hardiness cosegregates with the presence of this polypeptide or others induced during cold acclimation.

The same RNA isolations used for *in vitro* translations in this study were used in an attempt to create a cDNA library of cold-acclimated *Poncirus*. Differential screening of this library using radiolabeled RNA or cDNA from both cold-acclimated and nonacclimated *Poncirus* leaves may lead to the identification of cDNA clones that represent mRNA that increase in abundance upon cold acclimation. Such clones, if they detect RFLPs between *C. grandis* and *P. trifoliata*, would be obvious candidates for use in constructing a linkage map using interspecific progeny of these species. These progeny could also be screened for

Figure 3-1. Comparison of *in-vitro* translation products of RNA from leaves of nonacclimated and cold-acclimated *P. trifoliata*. A) Nonacclimated, B) cold acclimated. Arrows indicate major polypeptides induced during cold acclimation.


pI 7
IEF
pI 4
SDS
A
B

Figure 3-2. Comparison of *in-vitro* translation products of RNA from leaves of nonacclimated and cold-acclimated *C. grandis*. A) Nonacclimated, B) cold acclimated. Arrows indicate major polypeptides induced during cold acclimation.


pI 7
IEF
pI 4
A
SDS
B

cold hardiness and thus the mapping of QTLs involved in cold hardiness might be possible as well. This will be further discussed in Chapters 4 and 5.

CHAPTER 4
LINKAGE OF ISOZYMES AND RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN A BACKCROSS OF *CITRUS* AND *PONCIRUS*

Introduction

Genetic analysis in perennial fruit species has been hindered by several factors including large plant size, long juvenility periods, self- and cross-incompatibility, inbreeding depression, and apomixis (Janick and Moore, 1975; Moore and Janick, 1983). Considering that few Mendelian markers are known in these species, it is not surprising that genetic linkage maps have not yet been described for any tree fruit crop. The recent developments and applications of isozyme techniques in many plant species including fruit crops have increased the number of genetic markers available (Torres, 1983; 1989; Weeden, 1989). Isozymes have extended genetic maps in many species. In fruit crops, however, apart from a few cases where linkage between isozyme loci have been elucidated, linkage relationships among loci remain virtually unknown. The small number of isozymes analysed in most fruit crops preclude isozymes from being used as the sole basis for the creation of genetic linkage maps in these species.

Restriction fragment length polymorphisms (RFLPs) are being used extensively to create linkage maps in plants and animals. In plants, initially, RFLPs were used to further define the already extensive maps available in maize and tomato (Helentjaris et al., 1986; Bernatzky and Tanksley, 1986b). More

recently, RFLPs have allowed the initiation of linkage map construction in species where few prior linkage relationships existed, i.e., lentil (Havey and Muehlbauer, 1989), lettuce (Landry et al., 1987), pepper (Tanksley et al., 1988), and potato (Bonierbale et al., 1988; Gebhardt et al., 1989).

Citrus, in many respects, is attractive among fruit tree genera for conducting linkage analysis. It is a diploid with a relatively low haploid chromosome number, n=9 (Soost and Cameron, 1975), and small genome, 1C=.62 pg (Guerra, 1984). The genus appears to be highly heterozygous, and interspecific as well as intergeneric hybrids are easily made (Barrett, 1977; 1985). Genetic analysis of several isozyme systems have been reported (Torres et al., 1978; 1982) and initial studies have revealed two linkage groups comprised of five isozyme loci (Torres et al., 1985).

The goal of the research described in this chapter was to investigate the potential of combining isozyme analysis with RFLPs to create a linkage map in citrus.

Material and Methods

Plant Material

A BC_1 population was constructed using 'Pomeroy' trifoliate orange, *Poncirus trifoliata* (L.) Raf., as the donor parent and 'Thong Dee' pummelo, *Citrus grandis* (L.) Osbeck, as the recurrent parent. Pollen and budwood of the F_1 hybrid of these two species (selection 17-40 from a cross of 'Thong Dee' X 'Pomeroy') were obtained from Dr. Herb Barrett, USDA, Orlando, FL. Single trees were used as sources of pollen and budwood. Pollinations were made in

March, 1987, at the Florida Citrus Arboretum, Department of Plant Industries, Winter Haven, FL. Seeds were collected and germinated in December, 1987, and progeny plants were maintained in a greenhouse.

Isozyme analysis

Isozyme analysis was performed for 13 enzyme staining systems using previously described procedures (Durham et al., 1987; Moore and Castle, 1988). The enzymes analysed included adenylate kinase (AK, EC 2.7.4.3), creatine kinase (CK, EC 2.7.3.2), glutamate oxaloacetate transaminase (GOT, synonomous with aspartate aminotransferase, EC 2.6.1.1), glucose-6-phosphate dehydrogenase (G-6-PD, EC 1.1.1.49), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.4.0), peroxidase (PER, EC 1.11.1.7), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), phosphoglucose isomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), shikimate dehydrogenase (SkDH, EC 1.1.1.25), superoxide dismutase (SOD, EC 1.15.1.1), and triosephosphate isomerase (TPI, EC 5.3.1.1).

Rubisco SSU analysis

For analysis of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (SSU), leaf proteins were extracted into buffer (125 mM Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 4.6% SDS, and 20% glycerol) and electrophoresed in 10 or 15% polyacrylamide gels containing 1% SDS with buffer solutions as described by Laemmli (1970). For Western analysis, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Richmond, CA). The immunoassay was performed using standard procedures (Harlow and Lane, 1988)

with a polyclonal antibody raised against *Lemna* Rubisco SSU made available by Dr. Bruce O. Kohorn, Duke University, Durham, NC. The assay was performed without blocking and stained using an alkaline phosphatase conjugated 2° antibody (Protoblot Immunoscreening System, Promega Bioteck, Madison, WI).

Isolation, Digestion, and Blotting of Citrus DNA

DNA was isolated from citrus leaves using the method of Dellaporta et al. (1983) with modifications (for details see Appendix B). Digestion of DNA with restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, or *Pst*I, were according to manufacturer's instructions (Bethesda Research Laboratories, Gaithersburg, MD, or Boehringer Mannheim Biochemicals (BMB), Indianapolis, IN). Electrophoresis of digested DNA (1-2 μg/lane) was performed in 1.0% agarose gels in 1X TPE (80 mM Tris-phosphate, 2 mM EDTA) and DNA was transferred to nylon membranes (Hybond-N) according to manufacturer's instructions (Amersham, Arlington Heights, DE).

Source of Probes for RFLP Analysis

The probe designated pcPt was from a cDNA library constructed from mRNA isolated from cold-acclimated *Poncirus trifoliata* cv Pomeroy (a description of cold acclimation treatment and RNA isolation was presented in Chapter 3). mRNA was separated from total RNA using hybond MAP paper (Amersham) and cDNA synthesis was with a kit from BMB. The cDNA was ligated to *Eco*RI, 8-mer linkers (BMB), ligated into plasmid pTZ18R, and transformed into *E. coli* strain *TB1* according to Hanahan (1985). The library consisted of approximately 1,000 clones, the cloning efficiency was 3 X 10^5 clones/μg of insert.

Probes designated pRLc were provided by Dr. M. L. Roose, University of California, Riverside, and were from a cDNA library of mRNA isolated from leaves of rough lemon (*C. jambhiri* Lush). The probes designated pgCit were from a *Pst*I genomic library of 'Temple' tangor (*Citrus reticulata* Blanco X *C. sinensis* L. Osb.), constructed by Pan-chi Liou (Fruit Crops Department, University of Florida) during the course of this study. Details of this library construction are given in Liou (1990).

Radioactive Labeling and Hybridization of Probes

Cloned inserts used as probes were isolated using Geneclean (Bio 101, La Jolla, CA) following digestion with appropriate restriction enzymes and electrophoresis in 1% agarose, 1X TPE. Probes were labeled using a random primer labeling kit (BMB). Typical reactions included 100 ng of isolated insert and 40-50 μCi α-[^{32}P]dCTP (New England Nuclear, Boston, MA) resulting in specific activities of $\geq 10^8$ DPM per ug of insert. Unincorporated label was separated from probes by gravity flow exclusion chromatography through Sephadex G-50-50 (Sigma) as described in Maniatis et al. (1982). Blots to be probed were prehybridized (65°C, 2-24 hrs) and hybridized (65°C, 18-24 hrs) according to Church and Gilbert (1984). Following hybridization, blots were washed two times, 30 min each, 65°C, in 2X SSC (1X SSC is 150 mM NaCl, 15 mM NaCitrate, pH 7.0), 0.1% SDS and two times, 30 min each, 65°C, in 0.2X SSC, 0.1% SDS. Autoradiography was to Kodak X-omat AR X-ray film with intensifying screens (Dupont Cronex Lightning-Plus) at -80°C for one to four days. Probe removal was achieved by soaking in 0.4 N NaOH for 30 min at 45°C then

neutralizing in 0.2 M Tris-HCl pH 7.2, 0.1X SSC, 0.1% SDS for 30 min at 45°C. Blots were routinely reused six-eight times.

Linkage analysis of BC_1 segregation

Initial linkage analysis was performed by generating contingency tables of all pairwise combinations of loci using the FREQ procedure of SAS (SAS Institute Inc., Cary, NC). Loci were consider linked when p values were ≤ 0.005. The final linkage map was constructed using MAPMAKER (Lander et al., 1987; Whitehead Institute, Cambridge, MA) using defalt linkage criteria of LOD ≥ 2.0 and recombination fraction ≤ 0.4. Map distances were expressed in Kosambi (1944) centiMorgans (cM).

Results

Isozyme segregation

Of the 13 enzyme staining systems investigated, only seven (GOT, ME, MDH, IDH, PGD, PGM, and SkDH) gave well resolved banding patterns and exhibited genetically interpretable polymorphism within the backcross progeny population. The genetic analysis of several isozyme loci has been previously reported in citrus (Torres et al., 1978; 1982). Six of these isozyme loci (*Got1, Me1, Mdh2, Idh, Pgm,* and *Skdh*) were useful as genetic markers in the backcross population used in this study. Segregation analyses for these isozymes are given in Table 4-1. Segregation of PGD was also apparent in the progeny used for this study and is illustrated in Figure 4-1. Segregation of PGD has not been previously reported in citrus. Two zones of activity were evident: the faster migrating zone was designated PGD1, and the slower zone, PGD2. *C. grandis* exhibited a single dark

Table 4-1. Monogenic segregation of isozyme and RFLP loci in a backcross of *Citrus* and *Poncirus*. cc = homozygous for *Citrus* allele, cp = heterozygous for *Citrus* and *Poncirus* alleles.

Locus[a]	cc	cp	X^{2b}	Locus	cc	cp	X^2
			Isozymes				
Got1	49	14	19.44**	Pgd2	22	40	5.23*
Me1	45	18	11.57**	Pgm	32	31	0.02
Mdh2	47	16	12.94**	Skdh	39	24	3.57
Idh	32	31	0.02	SSU	32	26	0.62
Pgd1	36	27	1.29				
			RFLPs cDNA probes				
pRLc24	32	30	0.07	pRLc49	42	21	7.00**
pRLc25	32	30	0.07	pRLc60	49	15	18.06**
pRLc31	25	23	0.08	pRLc66L	37	27	1.56
pRLc32	35	22	2.97	pRLc89	35	27	1.03
pRLc38	40	22	5.23*	pRLc91	22	41	5.73*
pRLc39	32	30	0.07	pRLc94	21	42	7.00**
pRLc40	40	22	5.23*	pcPt001	38	26	2.25
pRLc41	29	32	0.15				
			RFLPs genomic probes				
pgCit005	40	23	4.59*	pgCit048	39	23	4.13*
pgCit009	36	28	1.00	pgCit049	40	18	8.35**
pgCit010	31	32	0.02	pgCit051	42	22	6.25*
pgCit011	27	37	1.56	pgCit052	27	38	1.86
pgCit012	29	36	0.75	pgCit054S	37	23	3.27
pgCit015	31	30	0.02	pgCit054L	29	31	0.67
pgCit017	42	22	6.25*	pgCit056	32	30	0.07
pgCit019	33	31	0.06	pgCit057L	33	31	0.06
pgCit021	37	27	1.56	pgCit061	38	27	1.86
pgCit027	37	27	1.56	pgCit062	32	29	0.15
pgCit028	27	32	0.51	pgCit063	27	30	0.16
pgCit030	18	42	9.60**	pgCit064	39	17	8.64**
pgCit035	37	24	2.77	pgCit065	40	24	4.00*
pgCit037	37	28	1.25	pgCit069	33	32	0.02
pgCit039	35	27	1.03	pgCit071	35	27	1.03
pgCit042	43	18	10.25**	pgCit074	40	16	10.29**
pgCit045	43	21	7.56**				

[a] For nomenclature of loci see 'Material and Methods'.
[b] *, ** indicate significant deviation from expected 1:1 ratio at p = 0.05 and 0.01, respectively.

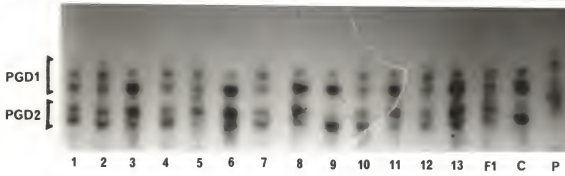


Figure 4-1. Banding patterns observed for isozymes of 6-phosphogluconate dehydrogenase in a backcross of *Citrus* and *Poncirus*. Lanes include extracts from *P. trifoliata* (P), *C. grandis* (C), their F_1 hybrid (F1), and backcross progeny (lanes 1-13). Genotypes of backcross progeny for *Pgd1*/*Pgd2* are: cp/cp in lanes 1, 2, 4, 5, 7, 10 and 12; cc/cp in lanes 3, 6, 8, 11, and 13; and cc/cc in lane 9. cc=homozygous for *C. grandis* allele, cp=heterozygous for *C. grandis* and *P. trifoliata* alleles. The origin of migration is at the bottom of the photograph and band migration is toward the anode.

band and a fainter, faster-migrating band at both the PGD1 and PGD2 regions while the F_1 exhibited three bands at PGD1 and two bands at PGD2. The banding pattern for *P. trifoliata* was unclear, however, the BC progeny all exhibited banding patterns that were similar to either *C. grandis* or the F_1 hybrid at either PGD1 or PGD2. This would be expected if separate loci were responsible for the polymorphism observed for PGD1 and PGD2 and *C. grandis* was homozygous at both loci and the F_1 was heterozygous at both loci. These loci were designated *Pgd1* and *Pgd2* in accordance with previous loci nomenclature in citrus (Torres et al., 1978, 1982, 1985). As demonstrated by progeny analysis (Figure 4-1, Table 4-1), both *Pgd1* and *Pgd2* appear to segregate as single loci, however, since the segregation of *Pgd2* exhibited significant skewing ($p \leq 0.05$) from the expected 1:1 ratio, additional segregating families need to be analyzed to confirm this interpretation of the data. Linkage analysis revealed that these loci are linked by a recombination distance of 26.4 cM.

SSU segregation

Polymorphism in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU) has been previously reported in citrus. Handa et al. (1986) were able to distinguish four forms of SSU in various species of citrus using isoelectric focusing (no individual plants had more than two forms of the enzyme). *P. trifoliata* exhibited a unique form of the enzyme as well. Roose et al. (1988) reported that in crosses between *Citrus* and *Poncirus*, the polymorphism in SSU exhibited monogenic inheritance based on data collected at both the protein and DNA level. Polymorphism between *C. grandis* and *P. trifoliata* was also observed in the present study in a protein of ca. 15 KDal (Figure 4-2),

presumably SSU, which segregated in a 1:1 ratio (Table 4-1). The identity of this protein as SSU was supported by its cross reactivity to a polyclonal antibody raised against *Lemna* SSU (Figure 4-2). Both *C. grandis* and *P. trifoliata* exhibited a single band with the *Citrus* band migrating slightly slower than the *Poncirus* band (Figure 4-2). The F_1 hybrid possesses both bands and the BC progeny were identical to either the F_1 hybrid, two-banded phenotype or the single band characteristic of *C. grandis*. Therefore, segregation of alleles at a single locus, designated *SSU*, appears to control the polymorphism observed between *C. grandis* and *P. trifoliata* for SSU.

Probe selection and RFLP analysis

Initially, blots containing DNA isolated from *P. trifoliata, C. grandis,* and the F_1 hybrid, restricted with enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, or *Pst*I, were hybridized with randomly selected clones from both the cDNA (pcPt) and genomic (pcCit) libraries. The cDNA clones, in general, exhibited a weaker hybridization signal than genomic clones in agreement with previous reports (Helentjaris et al., 1986). Furthermore, most of the cDNA clones exhibiting a strong hybridization signal produced unexpected banding patterns reminiscent of multiple copy or highly repetitive sequences (i.e., the hybridization signal was dispersed throughout the lanes or the hybridization signal was several orders of magnitude greater than would be expected from a single or low copy sequence). When polymorphisms were detected between the two plant species, the banding patterns tended to be complex. For these reasons only one of 21 clones (< 5%) from the pcPt library was useful as a probe for progeny RFLP analysis. The clones from a second cDNA library, designated pRLc, had been previously

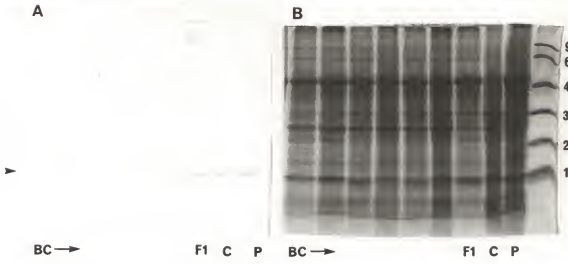


Figure 4-2. Western blot analysis and total protein stained gel revealing the segregation of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (SSU) in a backcross of *Citrus* and *Poncirus*. A.) Western blot analysis demonstrating immuno-crossreactivity of a 14 KDal protein (arrow) to an antibody raised against *Lemna* SSU. B.) Coomassie stained gel identical to A. Lanes include *P. trifoliata* (P), *C. grandis* (C), their F_1 hybrid (F1), and backcross progeny (BC). Genotypes of backcross progeny for *SSU* include, from left to right in both A and B, cc, cc, cp, cc, cp, and cp. cc=homozygous for *C. grandis* allele, cp=heterozygous for *C. grandis* and *P. trifoliata* alleles. Molecular weight standards (far-right-hand lane) are expressed in KDal.

selected as being useful to detect polymorphisms among different *Citrus* species (Roose, 1988). Of 20 pRLc clones tested, 14 (70%) proved to be useful for progeny RFLP screening.

The *Pst*I genomic library proved to be a much better source of useful probes. In other species, *Pst*I has been useful for genomic library construction because the enzyme does not cleave at restriction sites that are methylated. Since regions of the genome that are actively being transcribed are usually undermethylated (Burr et al., 1988), *Pst*I genomic libraries tend to be enriched for single or low copy number secquences suitable for RFLP analysis. In initial studies involving the pgCit clones, 11 of 18 probes (61%) detected a polymorphism between the two species that was potentially useful for progeny segregation analysis. Banding patterns produced by these genomic probes were less complex (fewer bands per lane) and thus more easily interpreted than patterns produced by cDNA probes. As expected, a few probes from the genomic library hybridized to repetitive sequences or sequences exhibiting maternal inheritance and were not further characterized.

Following the initial screening of 21 pcPt clones and 18 pgCit clones, further probe selection was performed only from the pgCit genomic library due to the higher percentage of useful polymorphisms detected by these probes. Furthermore, from the initial screening, it was determined that all polymorphisms detected between *C. grandis* and *P. trifoliata* were found when the DNA was restricted with either *Bgl*II, *Eco*RI, or *Eco*RV. Therefore, additional probe selections involved only these enzymes, see Figure 4-3.

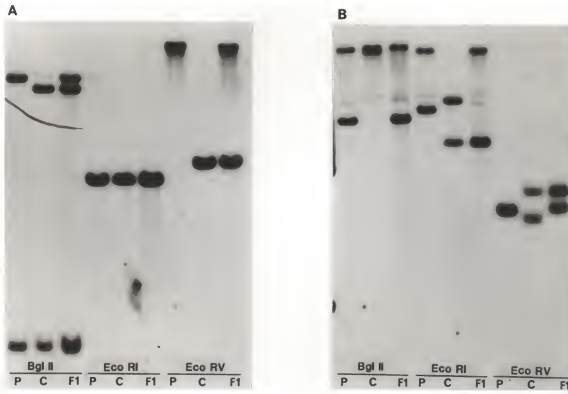


Figure 4-3. Southern blots of parental genotypes used to select useful clone/restriction enzyme combinations for detecting restriction fragment length polymorphisms (RFLPs) in a backcross of *Citrus* and *Poncirus*. Lanes are designated as containing DNA restricted with enzymes *Bgl*II, *Eco*RI, or *Eco*RV, isolated from *P. trifoliata* (P), *C. grandis* (C), or their F_1 hybrid (F1). Panel A was probed with clone pgCit035 which detected a polymorphism when the DNA was digested with either *Bgl*II or *Eco*RV, but not when digested with *Eco*RI. Panel B was probed with clone pgCit054S which detected a polymorphism with any of the three enzymes. In panel A, both *P. trifoliata* and *C. grandis* are homozygous for the RFLP while in panel B, *C. grandis* is heterozygous for the RFLP with any enzyme while *P. trifoliata* is heterozygous when restricted with either *Bgl*II or *Eco*RI.

During the probe selection analysis it became evident that *C. grandis* and *P. trifoliata* differed as to their level of heterozygosity but perhaps not as much as expected based on previously published isozyme data (Torres et al., 1978; 1982; 1985). Of the 48 DNA probes that detected a polymorphism between *Citrus* and *Poncirus*, based on the initial screening of these genotypes and their hybrid (for example, see Figure 4-3), 12 (25%) appeared to be polymorphic within *C. grandis*, while 18 (38%) were polymorphic within *P. trifoliata* (Table 4-2). That *C. grandis* was heterozygous at 25% of these loci is surprising since only low levels of heterozygosity have been previously reported at isozyme loci in this species (Torres et al., 1978; 1982; 1985). Also, *C. grandis* and *P. trifoliata* differed in the number of loci that were polymorphic with one out of three, two out of three, or three out of three restriction enzymes tested (Table 2). Data such as this have been used in rice as evidence that most polymorphisms in that species arise, not by base substitutions in restriction enzyme sequences, but as the result of insertions or deletions within the restriction fragment recognized by the probe (McCouch et al., 1988). The data presented in Table 4-2 were based on using only three restriction enzymes, however, they indicate that *C. grandis* and *P. trifoliata* may differ in the types of polymorphisms detected by DNA probes. In *C. grandis*, only three of 12 probes detected a polymorphism within the species with two or more restriction enzymes, contrasted with *P. trifoliata* where nine of 18 probes detected a polymorphism with two or more enzymes. In the F_1 hybrid, 39 of the 48 probes were polymorphic with two or more enzymes. The data seem to indicate that insertions/deletions accounted for less polymorphism in *C. grandis* than *P. trifoliata* while insertions/deletions accounted for a major portion of the

Table 4-2. Comparison of cDNA and genomic probes from Table 4-1 for detecting polymorphisms within *C. grandis*, *P. trifoliata*, and their F_1 hybrid and the number of restriction enzymes/probe that detect a polymorphism within each genotype.

	Citrus grandis	*Poncirus trifoliata*	*Citrus X Poncirus*
Total number of probes detecting polymorphism[a]	12 (25%)	18 (38%)	48 (100%)
Number of probes:			
-polymorphic with 3 restriction enzymes[b]	2	1	21
-polymorphic with 2 out of 3 enzymes	1	8	18
-polymorphic with 1 out of 3 enzymes	9	9	9

[a] Polymorphism in *Citrus* and *Poncirus* was based only on banding patterns exhibited during initial probe selection, no progeny segregation analyses were performed.

[b] Data based on analyses involving restriction enzymes *Bgl*II, *Eco*RI and *Eco*RV

polymorphism observed in the F_1 hybrid between these two species. This would suggest that such genome modifications have occurred after the evolutionary divergence of *Citrus* and *Poncirus*, further evidenced by the fact that only one probe, pgCit054S, was common to both *Citrus* and *Poncirus* in detecting a polymorphism with two or more restriction enzymes.

The clones that were selected as being potentially useful in detecting polymorphisms between *C. grandis* and *P. trifoliata* were used as markers in screening a backcross population between these species. Two general types of segregation were observed (Figure 4-4). When *C. grandis* was homozygous, the progeny segregated in a 1:1 ratio of the F_1 and *C. grandis* phenotypes (Figure 4-4, A). When *C. grandis* was heterozygous, the progeny exhibited four phenotypes and segregated in a 1:1:1:1 ratio (Figure 4-4, B). The cross was created using the presumably highly homozygous *C. grandis* as the maternal parent; thus few loci were expected to fall into this category. In fact, only seven loci exhibited a 1:1:1:1 ratio. The segregation at these loci was recoded such that only the segregation of the *P. trifoliata* allele was analysed (either its presence or absence) resulting in a transformation of the 1:1:1:1 ratio to a 1:1 ratio, see caption of Figure 4-4. Progeny receiving both alleles from *C. grandis* were considered to be homozygous, while progeny receiving one *Citrus* and one *Poncirus* allele were considered heterozygous. The observed segregation ratio for each probe is given in Table 4-1.

Several loci exhibited skewed segregation ratios. Of 57 total loci examined, 21 (37%) exhibited deviation from the expected 1:1 ratio at the $p=0.05$ significance level while 12 (21%) were also significant at $p=0.01$. Of the 21 loci

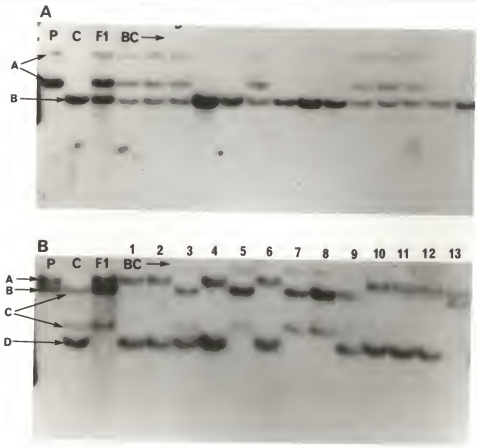


Figure 4-4. Southern blots illustrating the types of segregation observed during restriction fragment length polymorphism analysis in a backcross of *Citrus* and *Poncirus*. Genotypes in each lane include *C. grandis* (C), *P. trifoliata* (P), their F_1 hybrid (F1), and backcross progeny (BC, lanes 1-13). A) Hybridization pattern observed when DNA was restricted with *Bgl*II and probed with clone pgCit037. *P. trifoliata* is homozygous for the A allele (2 bands), *C. grandis* is homozygous for the B allele, the F_1 is heterozygous, AB, and the progeny segregate as either AB or BB.
B) Hybridization pattern observed when DNA was restricted with *Bgl*II and probed with clone pgCit052. *P. trifoliata* is heterozygous, AB, *C. grandis* is heterozygous, CD (allele C has 2 bands), the F_1 is AC, and the backcross progeny exhibit four segregation classes: AC in lane 13; AD in lanes 1, 2, 4, 6, and 10-12; CC in lanes 5, 7, and 8; and CD in lanes 3 and 9. For linkage analysis, the progeny genotypes were recoded to fit a 1:1 ratio by combining genotypes AC and AD (heterozygous with alleles from *P. trifoliata* and *C. grandis*) or CC and CD ("homozygous" for *C. grandis* alleles).

exhibiting skewedness, 17 showed an excess of *C. grandis* alleles, while only four showed an excess of *P. trifoliata* alleles.

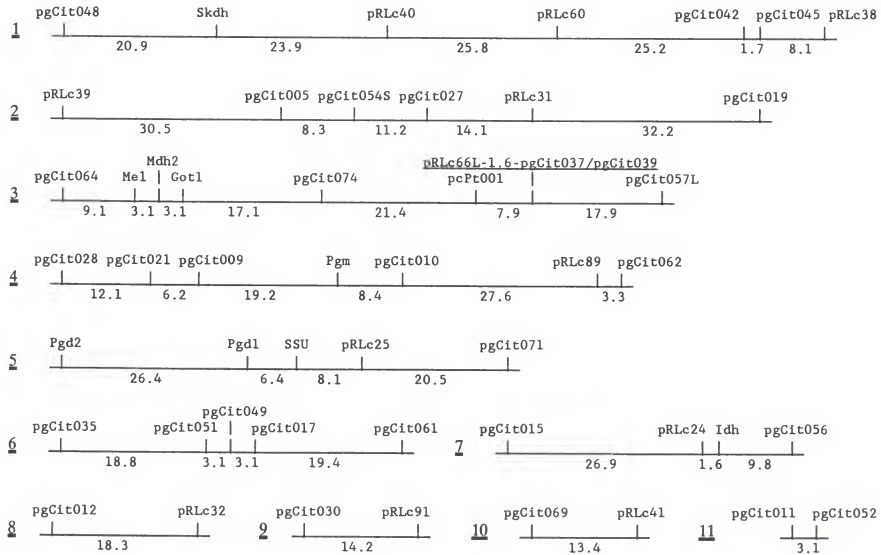
Linkage map

Analysis of the loci segregating in the backcross population using MAPMAKER revealed that 52 of these loci fall into 11 linkage groups, while five loci remain unassigned to any linkage group (Figure 4-5). The total length of the map illustrated in Figure 4-4 is 553 cM with the distance between markers ranging from 0 to 32.2 cM (mean distance of 13.5 cM).

Discussion

One aspect of the research presented in this chapter was the construction of a cDNA library from mRNA isolated from cold-acclimated leaf tissue of *P. trifoliata*. It was hoped that such a library could be used to identify RNA transcripts that may be induced during the cold-acclimation treatment. The construction of the library was difficult for unknown reasons; however, others working with citrus have also had problems creating both a cDNA library (M. L. Roose, personal communication) and a genomic library (P. C. Liou, personal communication). When a subset of the cDNA library constructed in this study was analysed, only a small fraction of the clones investigated (< 5%) showed potential for use in RFLP analysis. For this reason, further studies involving this cDNA library were abandoned. The research instead concentrated on selecting as many clones as possible from other available sources (the cDNA clones made available by M. L. Roose and the genomic library constructed by P. C. Liou).

Figure 4-5. Linkage map created from segregation analysis data collected from a backcross of *Citrus* and *Poncirus*. Linkage groups are designated by a number with double underlining. The values listed below each linkage group are recombination distances expressed as Kosambi (1944) centiMorgans (cM). The map displayed is the best possible fit of the data, however, certain regions of the map are ambiguous due to a lack of informative recombinations. In linkage group 1, the precise order of markers Sdh and pgCit048 in relation to pRLc40 and the precise order of markers pgCit042 and pgCit045 in relation to marker pRLc38 could not be unambiguously determined. Likewise, in linkage group 4, the precise order of markers pRLc89 and pgCit062 in relation to pgCit010 could not be determined, nor could the order of markers pRLc24 and Idh of linkage group 7 be determined. Five markers remain unlinked including, pgCit057L, pgCit063, pgCit065, pRLc49, and pRLc94.


1
pgCit048
Skdh
pRLc40
pRLc60
pgCit042
pgCit045
pRLc38
20.9
23.9
25.8
25.2
1.7
8.1
2
pRLc39
pgCit005
pgCit054S
pgCit027
pRLc31
pgCit019
30.5
8.3
11.2
14.1
32.2
3
Mdh2
pgCit064
Me1
Got1
pgCit074
pRLc66L-1.6-pgCit037/pgCit039
pcPt001
pgCit057L
9.1
3.1
3.1
17.1
21.4
7.9
17.9
4
pgCit028
pgCit021
pgCit009
Pgm
pgCit010
pRLc89
pgCit062
12.1
6.2
19.2
8.4
27.6
3.3
5
Pgd2
Pgd1
SSU
pRLc25
pgCit071
26.4
6.4
8.1
20.5
6
pgCit049
pgCit035
pgCit051
pgCit017
pgCit061
18.8
3.1
3.1
19.4
7
pgCit015
pRLc24
Idh
pgCit056
26.9
1.6
9.8
8
pgCit012
pRLc32
18.3
9
pgCit030
pRLc91
14.2
10
pgCit069
pRLc41
13.4
11
pgCit011
pgCit052
3.1

A large proportion (37%) of loci analysed in this study exhibited deviations from expected segregation ratios. This was not surprising since deviations from expected ratios have been previously reported for molecular markers, especially those analyzed in wide crosses (Weeden, 1989). In fact, Torres et al. (1985) observed skewed ratios for as many as seven of eight loci that they examined in certain families of a cross of *C. grandis* and *P. trifoliata*. Skewedness in segregation ratios of molecular and biochemical markers is thought not to result from pleiotrophy or epistasis, but to actually reflect abnormal segregation (Weeden, 1989). Zamir and Tadmore (1986) suggested that such distorted ratios may result from linkage to genes exposed to directional selection at either pre- or post-zygotic stages of development. Therefore, this skewedness does not prevent the use of these loci in mapping studies.

Torres et al. (1985) have previously reported 2 linkage groups in citrus. They reported the linkage of a MDH locus with two ME loci, *Mdh2*--17 cM--*Me01*--27 cM--*Me02*, and linkage of a GOT locus with a second MDH locus, *Got1*--7 cM--*Mdh1*. The results differ from those reported in this study. In this study, only one region of activity could be detected for ME and appeared to correspond to the locus described by Torres et al. (1985) as *Me1*. *Me1* was linked to a MDH locus and a GOT locus. Using the nomenclature desigated by Torres, the MDH locus was designated *Mdh2*; *Mdh1* was not segregating in this population. Likewise, the GOT locus analysed was designated *Got1*, while banding patterns were unclear in the GOT2 region. The linkage relationship among these loci is *Me1*--3.1 cM--*Mdh2*--3.1 cM--*Got1*. One reason for the ambiguity between these results and those of Torres et al. (1985) may be that certain isozymes of *Mdh1*

and *Mdh2* overlap in migration rates such that scoring for these loci is not always straight-forward. However, scoring was simplified in this study since *Mdh1* was not segregating. Also, segregation of the second GOT and ME loci has never been clear in the laboratory where these analyses were performed (G. A. Moore, personal communication), possibly due to differences in techniques used for isozyme analysis.

This study is one of the first to demonstrate the potential of combining RFLP and isozyme analysis for creating a genetic map in a tree fruit species. Similar analysis was performed by Liou (1990). Liou (1990) performed linkage analysis in an interspecific backcross of 'Clementine' mandarin and 'Duncan' grapefruit using 36 RFLP and six isozymes markers, where many of the markers were the same as those used in the present study. He found that 35 of these markers fell into eight linkage groups with a total map distance of 314 cM. The present study involved 48 RFLP and nine isozyme markers, resulting in 11 linkage groups with a total map distance of 553 cM. Since the number of linkage groups reported is greater than the number of haploid chromosomes of citrus (n=9), it is obvious that the genome has not been saturated with markers. Rather, it is likely that several of the smaller linkage groups would converge or join with larger linkage groups with the analysis of additional markers. Seven of the linkage groups reported contain four or more markers and four of these groups were greater than 75 cM in length. A conservative estimate would be that a 10 cM flanking region of the genome on either side of each of the seven smaller linkage groups (groups 5 through 11, Figure 4-4) as well as the five unlinked markers, could be included in the portion of the citrus genome covered by the current map. This

flanking region then would represent 240 additional cM of the genome for a total of 793 cM. Liou (1990) estimated the Citrus genome may span as much as 1,500 cM, thus, the current linkage analysis may cover as much as half of the genome.

One of the major conclusions reached by Liou (1990) was that insertions and deletions represent the major mechanism giving rise to RFLPs in citrus. The data from the present study suggest that insertions/deletions may be more common in some citrus species than others. However, these conclusions are based on analyses involving only three restriction enzymes while those of Liou (1990) were based on six enzymes and a similar study with rice by McCouch et al. (1988) was based on as many as 11 enzymes. Nevertheless, as expected, the genome of *C. grandis* appeared to be less heterozygous than that of *P. trifoliata* which is in agreement with previously reported isozyme data (Torres, 1978, 1982, 1985).

Genetic maps based on biochemical and molecular markers have proven useful in other species for mapping genes of economic importance including monogenic traits such as disease resistance and self-incompatibility alleles (Bournival et al., 1989; Landry et al., 1987; Sarfatti et al., 1989; Weeden, 1989; Young et al., 1988) as well as polygenic (quantitative) traits such as yield and cold tolerance (Martin et al., 1989; Nienhuis et al., 1987; Patterson et al., 1988; 1990; Weeden, 1989). In fruit tree species, long juvenility periods and large plant size combine to hinder conventional plant breeding by requiring large investments of time and land for progeny evaluation. Genetic maps in these species may provide the basis for early screening procedures (Tanksley et al., 1981) permitting breeders to make initial selections among very young progeny. Such selections

would not be made according to the progeny's phenotype, but according to its predicted phenotype based on determining the genotype of the progeny at biochemical or molecular markers loci known to cosegregate with a particular phenotype. It is in tree fruits where such analyses show the greatest potential for increasing the efficiency of cultivar improvement.

In the next chapter, the linkage map created in the backcross of *Citrus* and *Poncirus* will be used to map regions of the *Poncirus* genome that may influence cold hardiness.

CHAPTER 5
MAPPING GENES INVOLVED IN FREEZING TOLERANCE IN A BACKCROSS OF *CITRUS* AND *PONCIRUS* USING A LINKAGE MAP OF ISOZYMES AND RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

Introduction

The mapping of Mendelian factors underlying the expression of quantitative traits is possible using linkage maps that are densely populated with markers (Lander and Botstein, 1989). The advent of restriction fragment length polymorphisms (RFLPs) as genetic markers has made the creation of linkage maps possible in a wide range of species and has greatly refined the existing maps available in a few experimental organisms, such as tomato (Tanksley et al., 1989). Prior to the use of RFLPs, isozyme markers were sometimes used to map genes involved in the expression of quantitative traits (hereafter called QTLs, Geldermann (1975)). Much of this work was reviewed by Weeden (1989) and was usually conducted using either maize or tomato as experimental organisms. While many of the studies involving isozymes were able to detect putative linkage to QTLs, accurate mapping of QTLs was not possible because of the difficulty in arranging crosses having segregating markers densely spaced throughout the genome (Lander and Botstein, 1989). The abundance of RFLP markers available in many organisms overcomes this limitation.

Examples of the mapping of QTLs using isozymes and RFLPs can be drawn from research with tomato. The pioneering work was reported by Tanksley et al. (1982) who used an intergeneric backcross of tomato (*Lycopersicon esculentum* X *Solanum pennellii*), segregating for 12 isozyme loci, to identify QTLs involved in the expression of four quantitative traits: leaf ratio, stigma exsertion, fruit weight, and seed weight. They identified 21 chromosomal regions that influenced at least one of the traits investigated and QTLs having either a positive or negative effect on the quantitative traits investigated were identified. Pairwise combinations of loci, used to investigate epistatic interactions between QTLs, revealed 18 significant two-locus interactions for the traits studied. Epistatic interactions have been defined as deviations from additive gene action, i.e., when the phenotype resulting from an allelic substitution at one locus (AA vs Aa) is effected by allelic substitutions at one or more additional loci (Falconer, 1981). Similar analyses to those reported by Tanksley et al. (1982) were performed to investigate QTLs involved in cold-tolerance (Vallejos et al., 1983) and levels of 2-tridecadone, a naturally occurring insecticide (Zamir et al., 1984). Both studies used an interspecific backcross of *L. esculentum* X *L. hirsutum*. At least three QTLs were implicated in the expression of cold hardiness as were five conditioning the level of 2-tridecadone.

More recently, such analyses have involved the use of densely populated genetic linkage maps of isozymes and RFLPs. Patterson et al. (1988) used 70 genetic markers to investigate QTLs involved in the expression of soluble solids content, fruit mass, and fruit pH in an interspecific backcross of *L. esculentum* X

L. chmielewskii. They identified at least 16 QTLs that significantly influenced the expression of these traits.

Citrus cold hardiness is poorly understood genetically. Large differences in cold hardiness are evident among various citrus types; however, even greater differences are evident between *Citrus* and its close relatives, *Eremocitrus*, *Fortunella*, and *Poncirus* (Yelenosky, 1985). In general, crosses between *Citrus* and these related genera result in progeny that are intermediate in hardiness (Barrett, 1981), which suggests additive gene action. However, quantitative genetic analysis of cold hardiness in citrus is lacking.

The purpose of these experiments was to investigate: 1) the range in cold hardiness (specifically freezing tolerance) among backcross progeny of *Citrus grandis* X (*C. grandis* X *Poncirus trifoliata*); and 2) the possibility of identifying chromosomal regions that may influence cold hardiness.

Materials and Methods

Plant Material

The BC_1 population described in Chapter 4 was used as the source of plant material for the present study. Cuttings, consisting of either two or three nodes or stem tips, were taken from each BC_1 progeny and placed in a plastic flat (27.3 C 54.0 X 6.4 cm) containing artificial soil medium (Metromix 300, R. W. Grace). The flats were placed under intermittent mist (10 minute intervals during daylight hours) until roots had formed (approximately six weeks). The rooted cuttings were then transplanted to a six-inch pot with five to six cuttings of the same genotype placed in a pot. Additional cuttings were taken from genotypes that

failed to produce plants from the first round of propagation. After two rounds of propagation, 58 of the 65 BC_1 progeny had been successfully propagated. The plants were maintained in a greenhouse.

Cold Acclimation

Cold acclimation was accomplished under similar conditions as those described in Chapter 3. The plants, approximately six to eight months after transplanting, were transferred to a growth chamber where they were maintained at 5°C constant, 12 hour photoperiod, for four weeks. Following the first progeny freeze experiment, these plants were heavily pruned and returned to the greenhouse. After six weeks of regrowth, the plants were again cold acclimated under the same conditions used for the first progeny freeze experiment. Thus two growth flushes from the same plants were evaluated for cold hardiness: flush 1, which was six to eight months old when frozen and flush 2 which was six-weeks old when frozen.

Evaluation of Freezing Tolerance

The method for evaluating freezing tolerance was similar to that described in Chapter 3 except that detached leaves, rather than whole plants, were frozen, and post-freeze evaluation was solely on the basis of electrolyte leakage measurements.

Flush 1. For the first experiment, a total of 55 genotypes were evaluated for freezing tolerance at four temperature treatments (-8, -10, -12, and -14°C) with an unfrozen control. Fully expanded leaves from all plants of a particular genotype (grown in the same pot) were removed and grouped together. Three leaves for each treatment (a total of 15 leaves per genotype) were chosen in a semi-random

manner. The 15 leaves were ranked according to size; then, one each of the five largest leaves was randomly assigned to a treatment, one each of the five smallest leaves was randomly assigned to a treatment, and one each of the five remaining leaves was randomly assigned to a treatment. The reason for assigning leaves to treatments in this manner was to avoid any bias in leaf cold hardiness that might be associated with the leaf's relative position on the stem. The smaller leaves generally originated closer to the stem apex, while the larger leaves originated closer to the base of the stem. All the leaves selected for freezing originated from the same growth flush.

After being assigned to a treatment, the three leaves were together wrapped in a Kimwipe tissue, placed in a test tube, and the tissue was moistened with 2 ml of distilled water. A chip of ice was then added to each tube and the tubes were placed in a controlled-temperature, polyethylene glycol bath that had been equilibrated to 0°C. The temperature of the bath was then lowered at 2°C per hour and tubes were removed at -8, -10, -12, and -14°C. The tubes were placed at 4°C following removal from the bath to allow the leaves to slowly thaw overnight. Tubes representing the unfrozen control were placed at 4°C following the addition of the ice chip.

The following day, the previously frozen samples were prepared for electrolyte leakage evaluation. Leaves were removed from the tubes and samples were taken from each leaf using a #6 cork borer (1 cm inside diameter). Two leaf disks were removed from each leaf, each disk including the leaf midrib, and placed together into a sample vial containing 5 ml of distilled water. The samples were then evaluated as described in Chapter 3.

Flush 2. The protocol for the second experiment was nearly identical to the first experiment except that 52 genotypes were evaluated at six temperature treatments (-6, -8, -10, -12, -14, and -16°C), without an unfrozen control. The reason for the difference in plant numbers is that six plants from the first experiment did not produce enough leaves following regrowth to be included in the second experiment; however, 3 plants, not included in the first experiment because of a lack of leaves, produced enough leaves following regrowth to be included in the second experiment. Different temperature treatments were used in the two experiments for the following reasons. There appeared to be no significant differences among genotypes in electrolyte leakage for the unfrozen control in the flush 1 experiment; therefore, this treatment was eliminated from the flush 2 experiment. In flush 2, two additional temperature treatments were added: -6°C, because seven genotypes in flush 1 appeared to be severely damaged (i.e., had electrolyte leakage in excess of 50%) at the -8°C treatment, and -16°C, because 3 genotypes in flush 1 were not severely damaged (i.e., had electrolyte leakage less than 50%) at the -14°C treatment. Forty-nine genotypes were common to both flushes as were four temperature treatments (-8, -10, -12, and -14°C).

LT_{50} determination. The LT_{50} value for genotypes in flush 1 and flush 2 was considered to be the temperature where electrolyte leakage equalled 50%.

Statistical Analysis

To test whether individual chromosomal regions inherited from *P. trifoliata* were associated with the expression of freezing tolerance, the BC_1 progeny were grouped according to their genotype (i.e., either homozygous for *C. grandis* allele

or heterozygous for *C. grandis* and *P. trifoliata* alleles) and the average LT_{50} value of the genotypic groups were compared using a one-tailed T-test (TTEST procedure of SAS, SAS Institute, Cary, NC). A two-tailed T-test was also used to test for inequality of LT_{50} means for genotypic groups.

Results and Discussion

Response of Genotypes in Flush 1 vs. Flush 2

The LT_{50} distribution observed for the BC_1 progeny of *C. grandis* X (*C. grandis* X *P. trifoliata*) is illustrated in Figure 5-1. The overall response of flush 1 and flush 2 appeared to be similar; however, individual genotype responses in flush 1 and flush 2 were often quite different. Figure 5-2 illustrates a regression of the response of individual genotypes in flush 1 vs. flush 2. There was no correlation in response between flush 1 and flush 2 as evidenced by the R^2 value of 0.04, i.e., a correlation (r) of 0.2, which was statistically nonsignificant ($p=0.14$).

The reason for this disparity between flush 1 and flush 2 is unknown; however, the following possibilities may help to explain the phenomenon. First, these flushes varied greatly in age: flush 1 was six to eight months old when frozen, while flush 2 was only six-weeks old when frozen. The age of the flush may have had an impact on the ability of the plant to cold acclimate (i.e., increase in freezing tolerance when exposed to low, nonfreezing temperatures). Under field conditions, citrus trees are most cold hardy during winter when growth is arrested or quiescent (Yelenosky, 1985). An older growth flush is perhaps more apt to become quiescent when exposed to low temperature than a

Figure 5-1. Distribution of LT_{50} values observed in progeny of a backcross of *Citrus* and *Poncirus* following exposure to freezing temperatures. Fifty-five genotypes for flush 1 and 52 genotypes for flush 2 were frozen at temperatures ranging from -8 to -14°C for flush 1 and -6 to -16°C for flush 2, with three observations per temperature. The LT_{50} value was considered to be the temperature where electrolyte leakage equalled 50%.

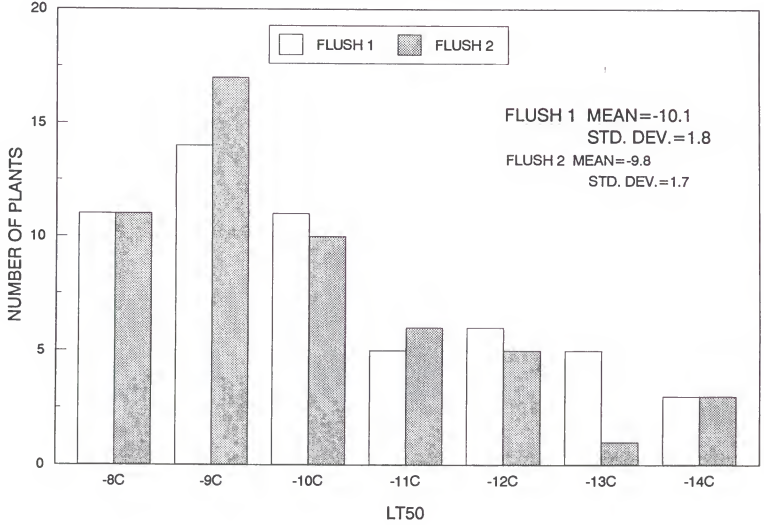
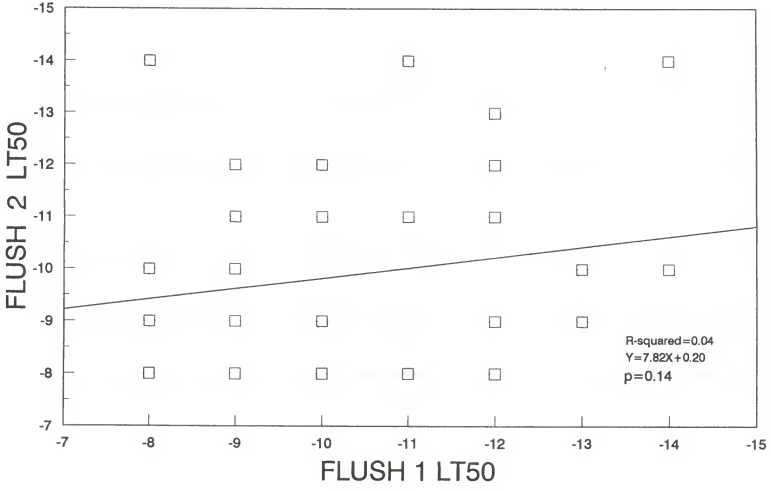

LT50 DISTRIBUTION FOR FLUSHES 1 AND 2
FLUSH 1
FLUSH 2
FLUSH 1 MEAN=-10.1
STD. DEV.=1.8
FLUSH 2 MEAN=-9.8
STD. DEV.=1.7
NUMBER OF PLANTS
20
15
10
5
0
-8C
-9C
-10C
-11C
-12C
-13C
-14C
LT50

Figure 5-2. A regression of response to freezing temperatures for individual genotypes from a backcross of *Citrus* and *Poncirus* for flush 1 vs. flush 2. Forty-nine genotypes were compared. The LT_{50} values were calculated as described in Figure 5-1.


REGRESSION OF RESPONSE OF FLUSH 1 ON FLUSH 2
FLUSH 2 LT50
-15
-14
-13
-12
-11
-10
-9
-8
-7
FLUSH 1 LT50
-7
-8
-9
-10
-11
-12
-13
-14
-15
R-squared=0.04
Y=7.82X+0.20
p=0.14

flush exposed to low temperature during active growth. Also, the accumulation of sucrose and proline are known to occur during cold acclimation (Yelenosky, 1985). A young growth flush may not have or be able to produce the photosynthate reserves needed to synthesize large quantities of such compounds. For these reasons, differences in response might be expected between flushes of different age. However, this alone cannot explain the differences observed between the flushes. If these were the only reasons, an overall difference in LT_{50} means between the young and old flush would be expected, which was not observed. One possibility that might not alter the population mean but could significantly change individual response would be if the rate of flush maturation was different among the progeny. In a backcross between *Citrus grandis*, a relatively vigorous citrus species, and *Poncirus trifoliata*, which is generally considered less vigorous, a difference in flush maturation rate, and likewise a difference in the ability to become quiescent or to partition stored photosynthate, might be expected. Therefore, this theory might explain some of the variation seen between genotypes in regards to freezing tolerance in flush 1 vs. flush 2.

Other factors were the overall health of the plants at the time they were subjected to controlled freezes and the time of year that the freeze test were performed. Flush 1 had been growing in a greenhouse for six to eight months prior to the freeze study. During that time, the plants became heavily infested with mites and certain genotypes may have been more susceptible to the mite foraging than others. The mite damage may have acted in some genotypes to induce stress responsive genes, which may have increased (or decreased) the freezing tolerance of certain genotypes. Also, the freezing experiment involving

flush 1 was conducted in early January, while flush 2 was subjected to freezing in late March. The relative daylength that the plants were exposed to in the greenhouse prior to cold acclimation may have influenced their ability to cold acclimate. Since *P. trifoliata* is deciduous, its ability to cold acclimate may be influenced by daylength which varied between these two dates. Therefore, a segregation for this daylength effect may account for certain disparities in individual genotype responses for flush 1 versus flush 2.

Clearly, additional experiments would be necessary to distinguish among these possibilities. The most important experiments would be to repeat the freeze tests involving flush 1 (without mite damage) and flush 2, to determine if similar results as these presently reported are obtained. Also, plant material from the maternal parent and the F_1 should be propagated and included in these experiments so that the heritability in response to freezing tolerance could be estimated. Because of the significant difference in response between flush 1 and flush 2, data for these two flushes were analyzed separately to investigate linkage of marker loci with QTLs affecting freezing tolerance.

Association of Individual Marker Loci with Freezing Tolerance

Differences in average LT_{50} values were observed when the BC_1 progeny were grouped according to their genotypes at marker loci. A one-tailed T-test was used to determine whether individual chromosomal regions inherited from *P. trifoliata* contained QTLs involved in the expression of freezing tolerance. The results of this analysis are given in Table 5-1. Four loci exhibited significant effects in flush 1, while only one locus exhibited a significant effect in flush 2. Since two of the loci, Pgd1 and SSU, mark areas on the same linkage group, it is

likely that they show linkage to the same QTL rather than separate QTLs, although these two alternatives cannot be distinguished with these data. Overall, it seems that three QTLs were identified that influenced freezing tolerance in flush 1, while only one QTL was identified that influenced freezing tolerance in flush 2. Again the reason for the disparity between flush 1 and flush 2 is unclear. A possibility might be that different genes condition freezing tolerance during different stages of leaf maturation. However, this does not explain why at least three chromosomal regions were shown to influence freezing tolerance in flush 1 while only one region was shown to influence freezing tolerance in flush 2. Should different freezing tolerance genes be active in flush 1 vs. flush 2, additional single locus effects unique to flush 2 would have been expected, unless many more genes, each with only a small effect, were active in the younger flush. Also, if certain stress responsive loci were induced during flush 1 by the mite foraging, this might explain why so many more individual chromosomal regions exhibited significant effects in flush 1 as opposed to flush 2. Flush 2 had no mite damage when the freeze test was conducted.

Other Loci Involved in Freezing Tolerance

When a two-tailed, rather than a one-tailed, T-test was used to test for significant differences in mean LT_{50} values between genotypic groups, additional loci that influenced freezing tolerance became apparent. The results of this analysis are presented in Table 5-2. Loci localized to two linkage groups, 1 and 2, and the unmapped locus, pRLc94, appeared to be linked to QTLs that influence freezing tolerance in flush 1. However, the heterozygous genotypic groups for these loci all correspond to decreased freezing tolerance when

Table 5-1. Individual marker loci which exhibited a significant effect for freezing tolerance in a backcross of *Citrus* and *Poncirus* using a one-tailed T-test. The T-test was used to test the hypothesis that genotypes heterozygous for the *Poncirus* and *Citrus* alleles were more freezing tolerant than genotypes homozygous for the *Citrus* allele. cc=segregation class homozygous for *Citrus* allele, cp=segregation class heterozygous for *Citrus* and *Poncirus* alleles. cc-cp=difference in means between the two segregation classes.

Locus[a]	Linkage group[b]	LT_{50}(°C)/n[c] cc	cp	cc-cp	*p*
		Flush 1			
pgCit064*	3	-9.8/35	-10.9/15	1.2	0.02
pgCit062	4	-9.8/28	-10.7/24	0.9	0.03
Pgd1	5	-9.8/33	-10.7/22	0.9	0.03
SSU	5	-9.7/31	-10.7/24	1.0	0.02
		Flush 2			
pgCit052	11	-9.4/25	-10.2/27	0.8	0.05

[a]* indicates loci showing deviation from expected 1:1 segregation ratio based on data presented in Table 4-1.
[b]From Chapter 4.
[c]LT_{50} is the temperature at which 50% electrolyte leakage occurs. n=the number of progeny from which the mean is calculated.

compared with the genotypic group homozygous for the *C. grandis* allele. One of these loci, pRLc60, also exhibited linkage to a QTL having a significant influence on freezing tolerance in flush 2. Other studies involving tomato have also detected QTLs that have an effect opposite that predicted by the phenotype of the parents. Tanksley et al. (1982) reported that QTLs having both positive and negative effects on stigma exsertion and leaf ratio were detected. They suggested that such QTLs may give rise to transgressive segregation, a situation in which certain progeny exhibit more extreme phenotypes than either parent. Because of a lack of freezing tolerance data regarding the parents of the backcross used in the present study, a similar conclusion cannot be reached in this study. However, it is apparent from Figure 5-1 that the phenotypic distribution for freezing tolerance among the backcross progeny exhibits greater variability from the mean in the more freezing tolerant direction. Some of the extremely freezing tolerant progeny may be transgressive; however, this conclusion is only speculative without freezing data from the F_1 and maternal parent of the backcross exposed to similar growth and cold acclimation conditions as the backcross progeny.

Another observation is that the majority of loci listed in Table 5-2 exhibit deviations from the expected 1:1 segregation ratio while this is true of only one locus in Table 5-1. The disproportionate number of progeny in one segregationclass may lead to a biased estimate of the mean LT_{50} value for that class, making the results of the T-test less reliable for those loci exhibiting skewed segregation ratios. Alternatively, the skewed segregation ratios exhibited by certain loci may be biologically significant in that such skewing may have resulted

Table 5-2. Individual marker loci which exhibited a significant effect for freezing tolerance in a backcross of *Citrus* and *Poncirus* using a two-tailed T-test. The T-test was used to test the hypothesis that the LT_{50} means for genotypic groups were unequal. Loci showing significant effects in Table 5-1 were not included in this analysis. cc=segregation class homozygous for *Citrus* allele, cp=segregation class heterozygous for *Citrus* and *Poncirus* alleles. cc-cp=difference in means between genotypic classes.

Locus[a]	Linkage group[b]	LT_{50}(°C)/n[c] cc	cp	cc-cp	p[d]
		Flush 1			
pRLc60*	1	-10.5/42	-8.9/13	1.6	0.001
pgCit042*	1	-10.6/36	-9.4/17	1.2	0.02
pgCit045*	1	-10.5/35	-9.5/20	1.0	0.03
pRLc38*	1	-10.5/33	-9.5/21	1.0	0.04
pgCit027	2	-10.7/31	-9.5/24	1.2	0.01
pRLc31	2	-10.9/23	-9.2/20	1.7	0.002
pRLc94*	?	-11.3/16	-9.7/38	1.6	0.007
		Flush 2			
pRLc60*	1	-10.1/39	-9.2/13	0.9	0.02

[a]* indicates loci showing deviations from expected 1:1 segregation ratio based on data presented in Table 4-1.
[b]From Chapter 4.
[c]LT_{50} is the temperature at which 50% electrolyte leakage occurs. n=the number of progeny from which the mean is calculated.
[d]Unequal variances were observed for the mean LT_{50} value of the two segregation classes for the locus pRLc60 in both flush 1 and flush 2. Therefore the Cochran approximation of the t-statistic was calculated for these data.

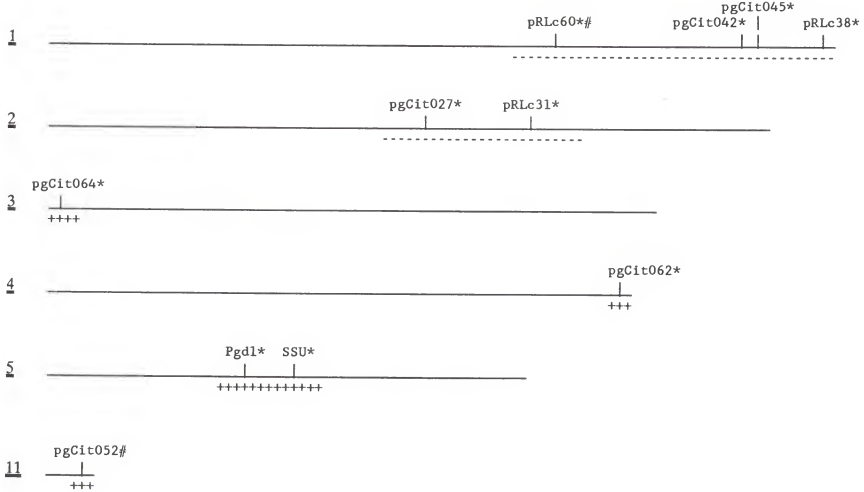
from linkage to genes exposed to directional selection, either prior to, or during, development of the zygote. Such genes may have a pleiotropic effect on freezing tolerance.

Map Location of QTLs

The position of loci linked to QTLs having either a positive or negative effect on freezing tolerance are illustrated in Figure 5-3. Loci exhibiting positive effects for freezing tolerance (i.e., increased freezing tolerance) were localized to four regions of the genome, including three regions for flush 1, one end of linkage group 3, one end of linkage group 4, and the central portion of linkage group 5, and a single region for flush 2 near locus pgCit052 on linkage group 11. Three regions of the genome exhibited negative effects in flush 1, including one end of linkage group 1, the central portion of linkage group 2, and the region represented by the unmapped locus, pRLc94. All regions exhibiting negative effects for freezing tolerance in flush 1 contained at least one locus that exhibited a highly significant (i.e., $p \leq 0.01$) effect. Locus pRLc60 showed linkage to a QTL with a negative influence on freezing tolerance in both flush 1 and flush 2.

The differences observed for the mean LT_{50} value of genotypic groups ranged from 0.9 to 1.2°C for those loci showing a positive effect (Tables 5-1) and from 1.0 to 1.7°C for those loci showing a negative effect (Table 5-2). Considering that the overall range in LT_{50} was from -8 to -14°C (a difference of 6°C), the range in variation explained by a single locus would be comparable to 0.9/6 to 1.2/6, or 15 to 20% for the loci showing a positive effect and 1.0/6 to 1.7/6 or 17 to 28% for the loci showing a negative effect. In these cases, the variation explained by a QTL was compared to the total variance observed for the backcross population.

Figure 5-3. Linkage map showing the position of marker loci which exhibited a significant effect for freezing tolerance in a backcross of *Citrus* and *Poncirus*. *, and # indicate markers which had a significant effect when analyzed individually in flush 1 or flush 2, respectively. The symbols under each linkage group indicate the positive (+ + + +) or negative (----) effect observed for each genomic region. The unlinked marker pRLc94 had a negative effect in both flushes.


1
pRLc60*#
pgCit042*
pgCit045*
pRLc38*
2
pgCit027*
pRLc31*
3
pgCit064*
++++
4
pgCit062*
+++
5
Pgd1*
SSU*
++++++++++++
11
pgCit052#
+++

It may be more informative to express this explained variation in relation to the variation seen between the parents of the backcross population rather than to the variation seen within the backcross population itself. Since freezing data is lacking for the parents, this calculation is not feasible; however, the variation between the parents is probably less than that seen withing the backcross progeny. Therefore, the percent variation explained by each QTL is probably underestimated.

Because many of the loci that exhibited a significant effect for freezing tolerance were linked to one another, it is probably incorrect to say that each marker locus is linked to at least one QTL. Rather, a conservative interpretation would be that each group of linked loci represents a QTL. Taking this into consideration, the data from Tables 5-1 and 5-2 can be interpreted to mean that at least four QTLs have been detected that influence freezing tolerance in a positive way (three in flush 1 and one in flush 2), while at least three QTLs have been detected that influence freezing tolerance in a negative way. The overall effects of the positive QTLs are less than the negative QTLs. This may be misleading, however, because the single locus analyses that were performed are not capable of distinguishing the difference between distant linkage between a marker locus and a QTL having a large effect, and close linkage between a marker locus and a QTL having a small effect (Lander and Botstein, 1989). Genetic recombination between the marker locus and the QTL causes the relative effect of the QTL to be underestimated. Also, the relative position of the QTL in relation to the marker locus can not be discerned using single locus

analysis. However, the gradation in significance of effect observed for certain linked loci may give some indication of the QTL's location. For example, a comparison of the relative significance exhibited by marker loci of linkage group 1 (Table 5-2, Figure 5-3) indicate that a QTL that negatively effects cold hardiness may be located closest to pRLc60. Also, the QTL is likely located on the pgCit042 side of pRLc60 since pRLc40, located on the other side of pRLc60, did not exhibit a significant effect for freezing tolerance.

Epistatic Interactions Among Loci

A two-way analysis of variance was used to test for epistatic interactions among QTL-linked loci and between QTL-linked loci and all other marker loci. The QTL-linked loci selected to include in the analysis were pgCit064, pgCit062, SSU, pRLc60, pRLc31, and pRLc94 from flush 1, and pgCit052 and pRLc60 from flush 2. These loci represented all linkage groups which exhibited an effect for freezing tolerance in either flush 1 or flush 2 (Tables 5-1 and 5-2). Epistatic interactions were not common considering the fact that only 5.6% of all possible combinations exhibited a significant interaction at $p=0.05$. Likewise, only 1.6% of all possible combinations of loci exhibited a significant interaction at $p=0.01$. Of the significant interactions at the $p=0.01$ level, approximately 50% involved comparisons between genotypic groups of disproportional progeny sizes. Often the mean on a genotypic group was based on fewer than 10 individuals. These small sample sizes may make such comparisons unreliable. Perhaps additional, more meaningful interactions would have been detected using a larger population.

In conclusion, certain of the QTLs detected by the individual locus analysis in this study (for example, the QTLs near pRLc60, pRLc31, pgCit062, and pRLc94)

individually appear to have accounted for over 15% of the variation seen for freezing tolerance within the backcross progeny. This may be an indication that such loci could be used to artificially select for freezing tolerance prior to an actual freeze test. However, this hypothesis would need to be tested by growing a larger population of progeny, genotyping them at these loci, and performing controlled freeze tests on the genotypic groups. Such an experiment would help to determine if the results of the present study are spurious, or if the loci identified in this study truly influence freezing tolerance.

CHAPTER 6
SUMMARY AND DISCUSSION

The overall purpose of this research was to construct a genetic linkage map in citrus and to use the map to identify quantitative trait loci (QTLs) that may influence citrus freezing tolerance. In Chapter 3, changes in gene expression following cold acclimation of *Citrus grandis* and *Poncirus trifoliata* were investigated. The translation profiles resulting from two-dimensional electrophoresis of *in-vitro* translation products revealed numerous changes between nonacclimated and cold-acclimated *Poncirus*, but few changes in similar studies involving *Citrus*. It was hoped that a cDNA library could be constructed from the RNA isolated from cold-acclimated *Poncirus*. Differential hybridization of this library to radiolabeled cDNA synthesized from RNA isolated from both cold-acclimated and nonacclimated plants may have revealed cDNA clones representing mRNAs induced during cold acclimation. Such clones, had they been identified, would have been used in RFLP analysis, and ultimately in the mapping of QTLs involved in freezing tolerance. However, the construction of the cDNA library was difficult, and when the library that was constructed was surveyed only one clone (of 21 tested) was useful in RFLP analysis. Because the library appeared to be such a poor source of probes for RFLP analysis, no differential screening of the library was attempted.

A genetic linkage map was constructed using both isozymes and RFLPs. The details of the map construction were presented in Chapter 4. The map was comprised of 11 linkage groups and covered a total of 553 cM. A saturation of the genome with marker loci was not achieved; however, approximately half of the citrus genome may have been mapped as a result of this study. This map will likely prove useful in the mapping of simply inherited traits in citrus. The mapping study was carried out using an intergeneric backcross between *Citrus grandis* and *Poncirus trifoliata*. Such a population would be expected to segregate for many traits of economic importance in citrus including resistance to certain pests (Citrus tristeza virus, *Phytophthora*, and nematodes) and mono- vs. polyembryony. It may be possible to map several of these traits with the extant progeny used in the mapping study. The mapping of polygenic traits may be possible as well.

Cold hardiness is a very important trait in citrus. In Chapter 5, studies were initiated to attempt mapping QTLs involved in citrus cold hardiness. Several putative QTLs were identified, including QTLs having either positive or negative effects. At least four QTLs were identified that influenced freezing tolerance in a positive way, while at least three QTLs were identified that influenced freezing tolerance in a negative way. Some loci individually accounted for at least 15% of the variability observed in freezing tolerance within the backcross population. It would be premature to predict whether these identified QTLs will be useful in breeding citrus for cold hardiness. For example, the disparity in results between growth flushes of different ages warrants further investigation. Also, it is unknown whether the QTLs identified in this study affect traits other than cold

hardiness, especially traits that might have adverse effects on fruit quality. The poor fruit quality of *Poncirus* is often inherited concurrently with cold hardiness. As the progeny used in this study mature, these plants could be evaluated for fruit characters as well. QTLs affecting fruit quality could be mapped to determine whether these loci fall close to those involved in cold hardiness, or whether selection for cold hardiness and against poor fruit quality might be possible by selecting for marker genotypes at the young seedling stage.

It is in perennial fruits where marker based selection at the seedling stage is particularly attractive. Fruit crops require large investments of land and time for the evaluation of progeny in a breeding program. If only potentially superior progeny, from among large segregating populations, could be selected at the seedling stage, the process of cultivar advancement in fruit crops would be greatly enhanced. Mapping economically important genes in these species using genetic linkage maps may make such selection schemes possible.

APPENDIX A
PROCEDURE USED FOR RNA ISOLATION

The following is the procedure (modified from Guy et al., 1985) used for isolating total RNA from leaves of *Citrus* and *Poncirus*.

1. Grind approximately 10 g of frozen (-80°C) leaf tissue in liquid N_2 to a fine powder. Transfer the powder to a 250 ml polypropyline centrifuge bottle.

2. Add 60 ml buffer P (0.1 M Tris-HCl pH 8.8, 0.1 M NaCl, 5 mM EDTA, 1% Na Sarkosyl, and 0.5 mg/ml or 40 units/ml heparin), shake vigorously for 2 to 3 minutes, and place on ice.

3. Add 30 ml buffer P' (buffer P without heparin) saturated phenol and 30 ml chloroform/isoamyl alcohol (24 parts chloroform:1 part isoamyl alcohol) and shake intermittently for 5 minutes. Place bottles on ice when not shaking. Spin bottles for 10 minutes, 4,500 x *g*, 5°C.

4. Remove organic phase to a clean bottle, add 20 ml of buffer P', shake vigorously 2 to 3 minutes, spin for 10 minutes, 4,500 x *g*, 5°C, and add aqueous phase to the previous prep.

5. Repeat phenol/chloroform extraction (step 3) 2 times, removing and discarding the organic phase each time.

6. Add 60 ml chloroform/isoamyl alcohol to aqueous phase, shake vigorously 1 to 2 minutes, and spin for 10 minutes, 4,500 x *g*, 5°C.

7. To aqueous phase add: a 1/10 volume of 3 M Na acetate pH 5.5/5 mM EDTA, 2 volumes of 95% ethanol, and mix by vortexing. Precipitate RNA at -20°C overnight.

8. Following precipitation, pellet RNA by spinning for 20 minutes, 10,000 x *g*, 5°C. Discard supernatant.

9. Resuspend pellet in 3 M Na acetate (20 ml/pellet) by vigorous shaking, and transfer to a baked, ribonuclease (RNase) free, glass corex centrifuge tube or phenol/chloroform washed polypropaline centrifuge tube. Further emulsify the pellet by vortexing or shaking. Repellet the RNA for 15

minutes at *g* max for corex or 10,000 x *g*. Remove and discard supernatant.

10. Repeat step 9, 2 to 3 times.

11. Dissolve RNA pellet in 8 to 10 ml of RNase free H_2O and spin briefly to pellet insoluble debris.

12. Transfer supernatant to a clean, RNase free tube, add a 1/10 volume of 3 M Na acetate/5 mM EDTA and 2 volumes of 95% ethanol, mix by vortexing. Allow RNA to precipitate overnight.

13. Pellet RNA for 20 minutes, 10,000 X *g*. Air dry the pellet and dissolve in a minimal volume (1 to 2 ml) of RNase free TE (10 mM Tris-HCl pH 7.6, 0.1 mM EDTA).

APPENDIX B
PROCEDURE USED FOR DNA ISOLATION

The following is the procedure (modified from Dellaporta et al., 1983) used for isolating large molecular weight DNA from leaves of citrus.

1. Grind approximately 1 g of young leaf material (or stem tips) to a fine powder in liquid nitrogen. Transfer powder with liquid nitrogen to a 45 ml centrifuge tube and place on ice.

2. After the liquid nitrogen has evaporated, add 15 ml of ice cold extraction buffer (0.1 *M* Tris-HCl pH 8.0, 50 m*M* EDTA pH 8.0, 0.5 *M* NaCl, 10 m*M* 2-mercaptoethanol) and 1 ml 20% SDS. Cap tube, shake vigorously, and incubate 10 minutes in a 65°C water bath.

3. Remove tubes from water bath and add 5 ml of 3 *M* potassium acetate (made according to Maniatis et al., 1982), shake tube vigorously, and place on ice for 20 minutes.

4. Remove tubes from ice and centrifuge at 25,000 x *g*, 5°C, for 20 minutes. Pour supernatant through 4 layers of Miracloth into a clean 45 ml tube and add 10 ml isopropanol. Mix by gently inverting the tube, incubate at 5°C overnight.

5. Allow tubes to warm slightly (5 minutes at room temperature) and pellet DNA at 20,000 x *g* for 15 minutes. Pour off supernatant and lightly dry pellet by inverting tubes on a paper towel for approximately 10 minutes.

6. Redissolve DNA with 0.5 ml of 50 m*M* Tris-HCl pH 8.0/10 m*M* EDTA and transfer, by pouring or a large bore pipet, to a 1.5 ml Eppendorf tube.

7. Add 0.5 ml phenol and mix by gently inverting the tube several times. Microfuge for 2 minutes. Extract aqueous (top) phase into a clean Eppendorf tube.

8. Repeat step 7 using 0.25 ml phenol and 0.25 ml chloroform/isoamyl alcohol (24:1) in place of phenol.

9. Repeat step 7 using 0.5 ml chloroform/isoamyl alcohol.

10. Add 1/2 volume of 7.5 *M* ammonium acetate and 1 ml absolute ethanol, mix by gently inverting the tube, and incubate at -20°C overnight.

11. Allow tubes to warm slightly and centrifuge at 12,000 x *g* for 15 minutes. Pour off ethanol and add 0.8 ml of 95% ethanol, cap tube and tap gently to dislodge pellet, let stand at room temperature for 15 minutes.

12. Centrifuge at 12,000 x *g* for 10 minutes. Pour off supernatant, vacuum dry pellet for 3 to 5 minutes (do not dry completely), and rehydrate in 0.1 to 0.2 ml of TE buffer (10 m*M* Tris-HCl pH 8.0/1 m*M* EDTA). Store DNA at -20°C.

APPENDIX C
GENOTYPES OF PROGENY AT MARKER LOCI

Sixty-five backcross progeny were scored for 57 loci including nine isozymes and 48 RFLPs. The locus name is listed first, followed by the genotype of each progeny. The progeny are listed in order from 1 thru 72 except that progenies 3, 8, 11, 21, 30, 48, and 67 were not included in the analysis. These progeny did not result from controlled pollinations but rather resulted from either selfing or outcrossing as evidenced by non-parental alleles being detected at some loci for these genotypes. Genotype A = plants homo- or heterozygous with only *Citrus grandis* allele(s) while genotype B = plants heterozygous with one *C. grandis* allele one *Poncirus trifoliata* allele. Missing data are designated with a hyphen. Most loci exhibited 1:1 segregation ratios typical of a backcross population; however, *C. grandis* was heterozygous for loci pcPt001, pgCit005, pgCit011, pgCit049, pgCit052, pgCit054S, and pgCit054L. These seven loci were recoded to reflect a 1:1 ratio as described in Chapter 4.

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*PGM      BBABBBAAAAAAAAABABB-BBABBBBBBA-BABAABABBABAAAAAAAAAABABBBAABBBBAABA
*MDH1     BBAAAABAAAAAAAAAAA-ABABAAABAA-AAAABABABAABBAAAAAAAAABAABAAAABBBAA
*PGD1     ABBAABAABABBABBBAA-BBBBBAAAAB-ABABAABAAABBAAAAAAAAABBBAAABBAAAABB
*PGD2     BBBBBBBBBBBBBBBBAB-BBBBBABAAA-ABABAABAA-BBAAAAABBAABBBAABBBAABBBB
*SDH      BABABABBBBBABBBAAA-AAAABABAAA-BAAABBAAAABAABABABABAABAAABAABAAAA
*IDH      AABABBBABBAAAAABBB-BABABABAAB-ABBAABABBABBABABAABBAABAAABBABBBAAB
*ME       BBABAABAAAAAAAAAAB-ABABAAABAA-AAAABABABAABBAAAAAAAAABAABAAAABBBAA
*GOT      ABAAAABAAAAAAAAAAA-ABABAAABAA-AAAABABABAABAAAAAAAAAABAABAAAABBBAA
*SSU      ABBAABBABAABABBBAA-B-BBBAA-AB-ABABBABAAA-BA-AAAAABABBBAAABBAAAAB-
*pcPt001  BBBAAAAABABAAABBBB-ABABAAAAABBABAABAAAABABAABABAAAAAABABBAAABBABBA
*pgCit005 BAB-BABABAAABBAAAAABBAAAAAAAABBBBBAAABBAAAAAAAAAAAAAABBBABBB-BAAA
*pgCit009 BAABBBAAAABAABBABBAABABBBABBAABABAABABBABAAAAAAAB-AAAABBABABBAABA
*pgCit010 BBAABBAAAAABAA-ABA-BBABBBBBBBBBABAABABBABAABAAAAAABABBBAABBBBAABA
*pgCit011 BBABABAABABBBAAABB-BBBBABAABAAAAABBBABBABBBBBBAAAABABBABBBBBBAAAB
*pgCit012 BAAAABABBBABBBABBAAAAAABAAAAABABBAAABAABBBBABAABBBBABBBBBBBBBBBA
*pgCit015 ABBABBBABBAABAB-BB---BABBBAABBABBABAABBABAABBAAAABAAABAABBBAAAAAA
*pgCit017 AABBAAABAABAAAAAAA-BABAABAABAAABBABABABAAAAAAAAAAABBBAABABBABBAAB
*pgCit019 BBABBBBABBABAABAAB-BAABBABABBBBABAABAAABBAAABABABBBBAABAAAAABBAAAA
*pgCit021 BAA-BBAAAABAABBAABAABABBBBBBBAABAABABBABAAAAAAABABAAABBABAABAABAA
*pgCit027 BAABBBBABBAABBAAAA-BBAABBABBBBBBBAAAABBAAAAAAAAAAAAAAABBBAAABABAAA
*pgCit028 BAABBBAAAABBAB--AB-ABBBBBBBBB-BABB-BABBABAAAAAAAAABA-ABBABABBBABA
*pgCit030 BBBBBABBBBABABBBBBBBBABBABAABBABBBBABABBABBBBBBAA--ABA-BB-ABA-BBAB
*pgCit035 AABAAAAABABAAABAAA-BABBAAABBA-ABBBBAABBBAA-BAABAAABABAABA-BABAAAB
*pgCit037 BBBAABAABBBAAABBBAABABAAAAAABABAABAAAABABAABABAAAAAAABBBABBBABBA
*pgCit039 BBBAABAABBBAAABBBAABABAAAAAABABAABAAAABABAABABA-AAAAABBBAB-BABB-
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*pgCit042   BABABAB-ABB-BA-ABA-AAAAABAAABAABAAAAAABBAABBAAAAABAAABAAAAAAAABAA
*pgCit045   BABABABBABBABABABA-AABAABAAABAABAAAAAABBAABBAAAAABAAABAAAAAAABAA
*pgCit048   BABBBAABBBBAABBAAAAAAABABAABBAAAAABAAAA--AABAABABABAABAAABABBAAA-
*pgCit049   AABBAAAAAABAAAAAAABBABAABAABAAABBABABABAAAAAAAAA-BBBAABAB---A---
*pgCit051   AABBAAAAAABAAAAAAA-BABBABAABAAABBABABABAAAABAAAAABBBAABABBABAAAB
*pgCit052   BBABABAABBBBBAAABBBBBBBABAAAAAAAABBBABABBBBBBAAAABABABBBBBBBAAAB
*pgCit054L  ABBBBBB-BA-AABBBAA---BBAABAABBABABBABABAAAAAABAABBBAABAABABABBBAB
*pgCit054S  BAAABABABBAABBAAAA-BBAABBAAAA-BBBAAAABB-AA-AAAAAAA-AABBBABBBABAAA
*pgCit056   AABABBBABBAAAAABBB-AAAABABAABBABBAABBBBABBA-A-AABBAAAABBBBABBAAB
*pgCit057L  BBBAABAABBBBABBBBB-AAABABBAAABBBAABAAAABAABABABBAAAAAABABABBAABBA
*pgCit061   BBBBAAABAABAABBAAABAABAABAABAAABBBABBBABAAAAAAAAABABBBABABAABBAAA
*pgCit062   BBAAABAABAABAA-ABA-BBABBBBB-ABBABAAAAABAABBBABAAAAABBBBBABBAA-BAA
*pgCit063   BBA-ABAAABBABB-BBB-ABBBAAB----BAAABBBABAAAABBBAAABAAAABBBBAB-BBA
*pgCit064   BBA-AABAAAAAAAA-AA-A-BBAAABAABAAAABAAABAABB----AAAAABBBBAAAA-BBAA
*pgCit065   ABBBBAAAABAAAAAAAA-AABBABAABABAAAABAAABBBAABBABAAAAABABABBBABAAAB
*pgCit069   BBABAAABABBABAAAABBABAAABBAABBBABABBBBBAAABAAAABBBABBBAAABAABBABA
*pgCit071   ABBAAABAAAAAAAABBB-ABBBBAAAABBABAABABAABBBAAABA-AB-BABAAABBAABABB
*pgCit074   BBA-AABAAABAAAB-AA---ABAAAAA-BAAAAB-ABAAABAAA-AAAAAABABBAAABB-BAA
*pRLc24     AABABBBABBAAAAABBB---BAAABAABBABBAABABBABABABAABBAAABAAABBABBAAB
*pRLc25     -BBAABBABAABABB-BA-BBBBBAAABBBABAAAABAABBBAAAAAAABABBBAAABBAAAAABB
*pRLc31     -AABBBBABBA-B---AA--------B-B-BBBAAAABBBBAA--AAAABBAABBAAAABBBAAA
*pRLc32     BAAAABABBBA-BB--BB-AAAAABAAA--BAABAAA-AABAABABAAAAAAABBBAABBA-BBA
*pRLc38     BABABAABABBABABABA---BABBAABAABBAAAAAABBAABBAAAAABAAABAAAAAAABAA
*pRLc39     BABAAABABAAABBABAA---AABBBBBABABBABAABBBABAAABAAAAAABABBBBBBBBAAAA
*pRLc40     BABABABBBBABBBBAAB---AAABAAABAAAAABAAAAABABAABBABAAAAAAAABAABAAAA
*pRLc41     BBABAAA-ABBAB-A-AB-AABBABBABBABABABBBBBABABAAAABBAABBBAAABBABBABA
*pRLc49     BAAAAABBABABBA-BAB-AABBABABAAABBBAAAAABABABABAAAAAAAAAAAAABAAABBA
*pRLc60     BABABAABBAABABABAA-AAAAABAAABAABABAAAAAABBABAAAAAABAAAAAAAAAAAAA
*pRLc66L    BBBAAAAABBBAAABBBB-ABABAAAAAABABAABAAAABABAABABAAAAAAABBBABBBABBA
*pRLc89     BBAAABAABAAAAABABA---ABBBBBBABBABAAAAABAABBBABAAAAABBBBAABBAABAA
*pRLc91     BBBABABBBBAABAABBBB-BBABBBABAABBABBBBAABBBABBBB-AABBAAABBBBABBBBAAB
*pRLc94     AABBBBBBBBABBABBBA-BABABBBABBAABBBAAABABBABBBBBBBBBBBBAABA-AAB
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APPENDIX D
FREEZING RESPONSE OF GENOTYPES AT ALL TEMPERATURE TREATMENTS

The individual response of genotypes at the various temperature treatments in flush 1 and flush 2 are expressed as percent electrolyte leakage. Each value is the mean of three observations. -- indicates missing data from genotypes not included in the freeze.

	Flush 1					Flush 2					
Genotype	0°C	-8°C	-10°C	-12°C	-14°C	-6°C	-8°C	-10°C	-12°C	-14°C	-16°C
1	09	22	50	54	70	--	--	--	--	--	--
2	08	17	62	79	76	--	--	--	--	--	--
4	10	24	66	75	84	08	16	56	75	76	76
5	17	80	84	89	91	13	28	46	72	84	84
6	14	49	66	69	81	10	21	51	69	75	81
7	11	43	72	82	85	20	36	54	54	60	71
9	13	38	70	79	79	17	49	64	86	83	89
10	11	45	71	79	80	28	67	60	78	79	80
12	07	37	59	70	71	11	35	68	71	78	79
13	09	59	64	77	75	10	20	49	66	82	80
14	08	49	55	75	72	10	19	35	33	54	69
15	--	--	--	--	--	08	18	36	70	73	73
16	10	54	85	86	87	16	28	63	73	77	79
17	08	36	68	81	83	14	38	69	76	75	80
18	09	44	52	59	89	16	40	66	72	77	73
19	11	44	60	73	67	11	17	47	68	73	75
20	12	21	45	67	63	14	46	69	72	78	77
22	17	26	44	56	67	--	--	--	--	--	--
23	--	--	--	--	--	--	--	--	--	--	--
24	12	46	35	54	64	10	17	35	44	54	66
25	--	--	--	--	--	--	--	--	--	--	--
26	07	48	40	50	64	--	--	--	--	--	--
27	09	20	29	47	70	11	40	49	50	58	68

28	09	28	56	69	64	10	53	75	80	82	85
29	08	33	51	71	75	12	42	60	69	71	88
31	13	37	54	68	65	14	43	61	67	77	75
32	--	--	--	--	--	--	--	--	--	--	--
33	12	47	67	78	76	15	57	74	77	83	85
34	18	35	47	82	77	11	61	67	81	84	73
35	--	--	--	--	--	--	--	--	--	--	--
36	07	37	32	64	69	16	27	45	56	70	72
37	10	33	67	87	81	11	43	71	83	86	84
38	07	31	67	74	70	25	16	36	52	66	65
39	09	34	34	62	89	08	54	65	77	80	78
40	10	26	62	45	76	10	19	40	66	86	90
41	12	16	37	43	61	09	30	63	73	83	79
42	--	--	--	--	--	13	58	72	84	76	84
43	11	35	68	74	80	08	32	61	74	77	80
44	06	70	74	80	85	13	39	63	71	82	83
45	16	52	88	84	88	12	25	70	67	81	75
46	--	--	--	--	--	--	--	--	--	--	--
47	09	18	31	47	46	11	36	58	68	84	91
49	11	42	67	76	89	17	54	79	85	86	86
50	--	--	--	--	--	--	--	--	--	--	--
51	11	16	19	40	59	--	--	--	--	--	--
52	14	44	55	69	76	14	18	44	51	69	80
53	09	23	24	39	68	10	17	69	57	80	89
54	09	15	51	87	79	12	26	45	51	76	89
55	11	32	25	68	56	13	12	17	31	48	58
56	08	54	72	75	75	13	43	62	75	81	87
57	08	30	62	76	78	10	27	57	77	82	93
58	12	41	64	77	78	32	56	74	83	82	85
59	09	13	19	37	60	09	34	57	74	79	87
60	11	11	17	38	51	14	18	25	31	52	66
61	11	15	47	56	63	07	24	38	77	84	84

62	12	25	52	47	68	10	33	66	71	82	86
63	11	53	31	62	74	12	15	36	61	79	86
64	11	10	20	25	32	11	22	52	65	57	69
65	15	12	24	27	41	--	--	--	--	--	--
66	08	45	81	91	95	09	29	69	80	81	83
68	--	--	--	--	--	10	17	23	54	61	66
69	18	22	36	90	90	09	42	64	74	79	81
70	12	19	28	40	58	12	45	60	73	77	85
71	10	17	22	46	65	11	53	62	67	74	76
72	--	--	--	--	--	--	--	--	--	--	--

LITERATURE CITED

Ashari, S., D. Aspinall, and M. Sedgley. 1988. Discrimination of zygotic and nucellar seedlings of five polyembryonic citrus rootstocks by isozyme analysis and seedling morphology. J. Hort. Sci. 63:695-703.

Barrett, H. C. 1977. Intergeneric hybridization of *Citrus* and other genera in citrus cultivar improvement. Proc. Int. Soc. Citriculture 2:586-589.

Barrett, H. C. 1981. Breeding cold-hardy citrus scion cultivars. Proc. Int. Soc. Citriculture 1:61-66.

Barrett, H. C. 1985. Hybridization of *Citrus* and related genera. Fruit Varieties J. 39:11-16.

Barrett, H. C., and A. M. Rhodes. 1976. A numerical taxonomic study of affinity relationships in cultivated *Citrus* and its relatives. Syst. Bot. 1:105-136.

Beckmann, J. S., and M. Soller. 1983. Restriction fragment length polymorphisms in genetic improvement: methodologies, mapping and costs. Thero. Appl. Genet. 67:35-43.

Beckmann, J. S., and M. Soller. 1986. Restriction fragment length polymorphisms and genetic improvement of agricultural species. Euphytica 35:111-124.

Ben-Hayyim, G., and Y. Goffer. 1989. Plantlet regeneration from a NaCl-selected salt tolerant callus culture of Shamouti orange (*Citrus sinensis* L. Osbeck). Pl. Cell Rep. 7:680-683.

Ben-Hayyim, G., A. Shani, and A. Vardi. 1982. Evaluation of isozyme systems in *Citrus* to facilitate identification of fusion products. Theor. Appl. Genet. 64:1-5.

Bernatzky, R., and S. D. Tanksley. 1986a. Majority of random cDNA clones correspond to single loci in the tomato genome. Mol. Gen. Genet. 203:8-14.

Bernatzky, R., and S. D. Tanksley. 1986b. Towards a saturated linkage map in tomato based on isozymes and random cDNA sequences. Genet. 112:887-898.

Bonierbale, M. W., R. L. Plaisted, and S. D. Tanksley. 1988. RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genet. 120:1095-1103.

Botstein, D., R. L. White, M. Skolnick, and R. W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Bournival B. L., J. W. Scott, and C. E. Vallejos. 1989. An isozyme marker for resistance to race 3 of *Fusarium oxysporum* f. sp. *lycopersici* in tomato. Theor. Appl. Genet. 78:489-494.

Bringhurst, R. S. 1983. Breeding strategy, pp 147-153. In: J. N. Moore and J. Janick (eds.), Methods in Fruit Breeding. Purdue Univ. Press, West Lafayette, IN.

Burr, B. F. A. Burr, K. H. Thompson, M. C. Albertson, and C. W. Stuber. 1988. Gene mapping with recombinant inbreds in maize. Genetics 118:519-526.

Burr, B., S. V. Evola, F. A. Burr, and J. S. Beckmann. 1983. The application of restriction fragment length polymorphism to plant breeding, pp 45-59. In: J. K. Setlow and A. Hollaender (eds.), Genetic Engineering: Principles and Methods. Plenum Press, New York and London.

Button, J., A. Vardi, and P. Speigel-Roy. 1976. Root peroxidase isoenzymes as an aid in *Citrus* breeding and taxonomy. Theor. Appl. Genet. 47:119-123.

Cameron, J. W., and H. B. Frost. 1968. Genetics, breeding, and nucellar embryony, pp 325-389. In: W. Reuther, L. D. Batchelor, and H. J. Weeber (eds.), The Citrus Industry, Vol. II. University of California, Berkeley, CA.

Cameron, J. W., and R. K. Soost. 1979. Sexual and nucellar embryony in F_1 hybrids and advanced crosses of *Citrus* with *Poncirus*. J. Am. Soc. Hort. Sci. 104:408-410.

Church, G. M., and W. Gilbert. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. U.S.A. 81:1991-1995.

Cooper, W. C., P. C. Reese, and J. R. Furr. 1962. Citrus breeding in Florida--past, present and future. Proc. Fla. State Hort. Soc. 75:5-13.

Dellaporta, S. L., J. Wood, and J. B. Hicks. 1983. A plant DNA minipreparation: version II. Pl. Mol. Biol. Rep. 1:19-21.

Donis-Keller, H., P. Green, C. Helms, S. Cartinhour, B. Weiffenbach, K. Stephens, T. P. Keith, D. W. Bowden, D. R. Smith, E. S. Lander, D. Botstein, G. Akots, K. S. Rediker, T. Gravius, V. A. Brown, M. B. Rising, C. Parker, J. A.

Powers, D. E. Watt, E. R. Kauffman, A. Bricker, P. Phipps, H. Muller-Kahle, T. R. Fulton, S. Ng, J. W. Schumm, J. C. Braman, R. G. Knowlton, D. F. Barker, S. M. Crooks, S. E. Lincoln, M. J. Daly. 1987. A genetic linkage map of the human genome. Cell 51:319-337.

Durham, R. E., G. A. Moore, and W. B. Sherman. 1987. Isozyme banding patterns and their usefulness as genetic markers in peach. J. Am. Soc. Hort. Sci. 112:1013-1018.

Esen A., and R. W. Scora. 1977. Amylase polymorphism in citrus and some related genera. Am. J. Bot. 64:305-309.

Esen, A., and R. K. Soost. 1976. Peroxidase polymorphism in *Citrus*. J. Hered. 67:199-203.

Falconer, D. S. 1981. Introduction to Quantitative Genetics, Second Edition. Longman, Inc. and Wiley and Sons, Inc., New York.

Frost, H. B., and R. K. Soost. 1968. Seed reproduction: development of gametes and embryos, pp 290-324. In: W. Reuther, L. D. Batchelor, and H. J. Weeber (eds.), The Citrus Industry, Vol. II. University of California, Berkeley, CA.

Gebhardt, C., E. Ritter, T. Debener, V. Schachtschabel, B. Walkemeier, H. Uhrig, and F. Salamini. 1989. RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor. Appl. Genet 78:65-75.

Geldermann, H. 1975. Investigations on inheritance of quantitative characters in animals by gene markers. I. Methods. Theor. Appl. Genet. 46:319-330.

Gilmour, S., R. K. Hajela, and M. F. Thomashow. 1988. Cold acclimation in *Arabidopsis thaliana*. Pl. Physiol. 87:745-750.

Goodman, M. M., and C. W. Stuber. 1983. Maize, pp.1-56. In: S. D. Tanksley and T. J. Orton (eds.), Isozymes in Plant Genetics and Breeding, Part 1B. Elsevier, Amsterdam.

Grosser, J. W., and F. G. Gmitter. 1990. Somatic hybridization of *Citrus* with wild relatives for germplasm enhancement and cultivar development. HortScience 25:147-151.

Guerra, M. S. 1984. Cytogenetics of Rutaceae. II. Nuclear DNA content. Caryologia 37:219-226.

Guy, C. L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu. Rev. Pl. Physiol. Mol. Biol. 41:187-223.

Guy, C. L., and D. Haskell. 1987. Induction of freezing tolerance in spinach is associated with the synthesis of cold acclimation induced proteins. Pl. Physiol. 84:872-878.

Guy, C. L., and D. Haskell. 1988. Detection of polypeptides associated with the cold acclimation process in spinach. Electrophoresis 9:787-796.

Guy, C. L., D. Haskell, and G. Yelenosky. 1988. Changes in freezing tolerance and polypeptide content of spinach and citrus at 5°C. Cryobiology 25:2264-271.

Guy, C. L., R. L. Hummel, and D. Haskell. 1987. Induction of freezing tolerance in spinach during cold acclimation. Pl. Physiol. 84:868-871.

Guy, C. L., K. J. Niemi, and R. Brambl. 1985. Altered gene expression during cold acclimation of spinach. Proc. Natl. Acad. Sci. USA 82:367-677.

Hanahan, D. 1985. Techniques for transformation of *E. coli*, pp 109-135. In: D. M. Glover (ed.), DNA Cloning Volume 1, A Practical Approach. IRL Press, Oxford, England.

Handa, T., Y. Ishizawa, and C. Oogoki. 1986. Phylogenetic study of fraction 1 protein in the genus *Citrus* and it close related genera. Jap. J. Genet. 61:15-24.

Hansche, P. E. 1983. Response to selection, pp 154-171. In: J. Janick and J. N. Moore (eds.), Methods in Fruit Breeding. Purdue Univ. Press, West Lafayette, IN.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Havey, M. J., and F. J. Muehlbauer. 1989. Linkages between restriction fragment length, isozyme, and morphological markers in lentil. Theor. Appl. Genet. 77:395-401.

Hearn, C. J. 1973. Development of scion cultivars of citrus in Florida. Proc. Fla. State Hort. Soc. 86:84-88.

Hearn, C. J. 1979. 'Sunburst' citrus hybrid. HortScience 14:761-762.

Hearn, C. J. 1985. Citrus scion improvement program. Fruit Varieties J. 39:34-37.

Hearn, C. J. 1986. Development of seedless grapefruit cultivars through bud wood irradiation. J. Am. Soc. Hort. Sci. 111:304-306.

Helentjaris, T. 1987. A genetic linkage map for maize based on RFLPs. Trends in Genet. 3:217-221.

Helentjaris, T., and B. Burr (eds.). 1989. Development and Application of Molecular Markers to Problems in Plant Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Helentjaris, T., G. King, M. Slocum, C. Siedenstrang, and S. Wegman. 1985. Restriction fragment polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. Pl. Mol. Biol. 5:109-118.

Helentjaris, T., M. Slocum, S. Wright, A. Schaefer, and J Nienhuis. 1986. Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor. Appl. Genet. 72:761-769.

Hensz, R. A. 1981. Bud mutations in citurs cultivars in Texas. Proc. Int. Soc. Citriculture 1:89-91.

Hirai, M., and I. Kajiura. 1987. Genetic analysis of leaf isozymes in citrus. Japan. J. Breed. 37:377-388.

Hirai, M., I. Kozaki, and I. Kahura. 1986a. Isozyme analysis and phylogenic relationship of citrus. Japan. J. Breed. 36:377-389.

Hirai, M., I. Kozaki, and I. Kajiura. 1986b. The rate of spontaneous inbreeding of trifoliate orange and some characteristics of the inbred seedlings. Japan. J. Breed. 36:138-146.

Hodgson, R. W. 1967. Horticultural varieties of citrus, pp 431-591. In: W. Reuther, H. J. Weeber, and L. D. Batchelor (eds.), The Citrus Industry, Vol. I. University of California, Berleley, CA.

Hunter, R. L., and C. L. Markert. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science 125:1294-1295.

Hutcheson, C. E., and W. J. Wiltbank. 1970. Cold hardiness of selected citrus varieties as determined by freezing detached leaves. Proc. Fla. State Hort. Soc. 83:95-98.

Hutchison, D. J. 1974. Swingle citrumelo--a promising rootstock hybrid. Proc. Fla. State Hort. Soc. 87:89-91.

Ikeda, I. 1982. Freeze injury and protection of citrus in Japan, pp 575-589. In: P. H. Lee and A. Sakai (eds.), Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications, Vol. 2. Academic Press, New York.

Iglesias, L., H. Lima., and J. P. Simon. 1974. Isoenzyme identification of zygotic and nucellar seedlings in *Citrus*. J. Hered. 65:81-84.

Janick, J., and J. N. Moore (eds.). 1975. Advances in Fruit Breeding. Purdue Univ. Press, West Lafayette, IN.

Khan, I. A., and M. L. Roose. 1988. Frequency and characteristics of nucellar and zygotic seedlings in three cultivars of trifoliate orange. J. Am. Soc. Hort. Sci. 113:105-110.

Kosambi, D. D. 1944. The estimation of map distances from recombination values. Ann. Eugen. 12:172-175.

Kurkela, S., M. Franck, P. Heino, V. Lang, and E. T. Palva. 1988. Cold induced gene expression in *Arabidopsis thaliana* L. Pl. Cell Rep. 7:495-498.

Laemmli, U. L. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lander, E. S., and D. Botstein. 1989. Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. Genet. 121:185-199.

Lander, E. S., P. Green, J. Abrahamson, A. Barlow, M. J. Daly, S. E. Lincoln, and L. Newburg. 1987. Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181.

Landry, B. S., R. V. Kesseli, B. Farrara, and R. W. Michelmore. 1987. A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. Genet. 16:331-337.

Landry, B. S., and R. W. Michelmore. 1985. Selection of probes for restriction fragment length analysis from plant genomic clones. Pl. Mol. Biol. Rep. 3:174-179.

Landry, B. S., and R. W. Michelmore. 1987. Methods and applications of restriction fragment length polymorphism analysis to plants, pp 25-44. In: G. Bruning, T. Kosuge, J. Harada, and A. Hollaender (eds.), Tailoring Genes for Crop Improvement: An Agricultural Perspective. Plenum Press, New York.

Lang, V., P. Heino, and E. T. Palva. 1989. Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. 77:729-734.

Levitt J. 1980. Responses of Plants to Environmental Stresses. I. Chilling, Freezing and High Temperature Stresses, Ed. 2. Academic Press, Orlando.

Lisenfeld, D. R., D. L. Auld, G. A. Murray, and J. B. Swensen. 1986. Transmittance of winterhardiness in segregating populations of peas. Crop Sci. 26:49-54.

Liou, P. C. 1990. A molecular study of the citrus genome through restriction fragment length polymorphism and isozyme analysis. Doctoral Dissertation. University of Florida, Gainesville, Florida.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Martin, B., J. Nienhuis, G. King, and A. Schaefer. 1989. Restriction fragment length polymorphisms associated with water use efficiency in tomato. Science 243:1725-1728.

McCouch, S. R., G. Kochert, S. H. Yu, Z. Y. Wang, G. S. Khush, W. R. Coffman, and S. D. Tanksley. 1988. Molecular mapping of rice chromosomes. Theor. Appl. Genet. 76:815-829.

Moore G. A., and W. S. Castle. 1988. Morphological and isozymic analysis of open-polinated citrus rootstock populations. J. Hered. 79:59-63.

Moore, J. N., and J. Janick (eds.). 1983. Methods in Fruit Breeding. Purdue Univ. Press, West Lafayette, IN.

Nielsen, G. 1985. The use of isozymes as probes to identify and label plant varieties and cultivars. Isozymes: Current Topics in Biological and Medical Research 12:1-32.

Nienhuis, J., T. Helentjaris, M. Slocum, B. Ruggero, and A. Schaefer 1987. Restriction fragment length polymorphism analysis of loci associated with insect resistance in tomato. Crop. Sci. 27:797-803.

O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis. J. Biol. Chem. 250:4007-4021.

Ortiz, J. M., J. L. Tades, J. Guerri, and J. B. Forman. 1981. Distinction between hybrid and nucellar citrus trees by analysis of their biochemical compounds. Proc. Int. Soc. Citruculture 1:4-7.

Osborn, T. C., D. C. Alexander, and J. F. Fobes 1987. Identification of restriction fragment length polymorphisms linked to genes controlling soluble solids content in tomato fruit. Theor. Appl. Genet. 73:350-356.

Parlevliet, J. E., and J. W. Cameron. 1959. Evidence on the inheritance of nucellar embryony in *Citrus*. Proc. Am. Soc. Hort. Sci. 74:252-260.

Parsons, L. R., T. A. Wheaton, and D. H. P. Tucker. 1986. Florida freezes and the role of water in citrus cold protection. HortScience 21:2.

Paterson, A. H., J. W. DeVerna, B. Lanini, and S. D. Tanksley. 1990. Fine mapping of quantitative trait loci using selected overlapping recombinant chromosomes, in an interspecies cross of tomato. Genet. 124:734-742.

Paterson, A. H., E. S. Lander, J. D. Hewitt, S. Peterson, S. E. Lincoln, and S. D. Tanksley. 1988. Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature 335:721-726.

Peirce, L. C., and J. L. Brewbaker. 1973. Applications of isozyme analysis in horticultural science. HortScience 8:17-22.

Prakash, S., R. C. Lowontin, and J. L. Hubby. 1969. A molecular approach to the study of genic heterozygosity in natural populations. IV. Patterns of genetic variation in central, marginal, and isolated populations of *Drosophila pseudoobscura*. Genet. 61:841-858.

Rick, C. M. 1983. Tomato (*Lycopersicon*), pp 147-165. In: S. D. Tanksley and T. J. Orton (eds.), Isozymes in Plant Genetics and Breeding, Part 1B. Elsevier, Amsterdam.

Rick, C. M., and J. F. Fobes. 1974. Association of an allozyme with nematode resistance. Rept. Tomato Genet. Coop. 24:25.

Roose, M. L. 1988. Isozymes and DNA restriction fragment length polymorphisms in citrus breeding and systematics, pp 57-67. In: R. Goren and K. Mendel (eds.), Proceedings of the Sixth International Citrus Congress. Margraf Scientific Books, Weikersheim, West Germany.

Roose, M. L., and S. N. Traugh. 1988. Identification and performance of citrus trees on nucellar and zygotic rootstocks. J. Am. Soc. Hort. Sci. 113:100-105.

Roose, M. L., M. L. Gottlieb, and S. D. Traugh. 1988. A single gene specifies the small subunit of ribulose-1,5-bisphosphate carboxylase in citrus. Genome 30, suppl. 1:452.

Robertson, D. S. 1989. Understanding the relationship between qualitative and quantitative genetics, pp 81-87. In: T. Helentjaris and B. Burr (eds.), Development and Application of Molecular Markers to Problems in Plant Breeding. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sarfatti, M., J. Katan, R. Fluke, and D. Zamir. 1989. An RFLP marker in tomato linked to *Fusarium oxysporum* resistance gene *I2*. Theor. Appl. Genet. 78:755-759.

Sax, K. 1923. The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris*. Genet. 8:552-560.

Scora, R. W., J. Kumamoto, R. K. Soost, and E. M. Nauer. 1982. Contribution to the origin of the grapefruit, *Citrus paradisi* (Rutaceae). Syst. Bot. 7:170-177.

Shields, C. R., T. J. Orton, and C. W. Stuber. 1983. An outline of general resource needs and procedures for the electrophoretic separation of active enzymes from plant tissues, pp 443-468. In: S. D. Tanksley and T. J. Orton (eds.), Isozymes in Plant Genetics and Breeding, Part A. Elsevier, Amsterdam.

Simmons, N. W. 1979. Principles of Crop Improvement. Longman Inc., New York.

Singh, J., and A. Laroche. 1988. Freezing tolerance in plants: a biochemical overview. Biochem. Cell Biol. 66:650-657.

Smithies, O. 1955. Zone electrophoresis in starch gels. Biochem. J. 61:629-641.

Soller, M., and J. S. Beckmann. 1983. Genetic polymorphism in varietal identification and genetic improvement. Theor. Appl. Genet. 67:25-33.

Soost, R. K., and J. W. Cameron. 1975. Citrus, pp 507-540. In: J. Janick and J. Moore (eds.), Advances in Fruit Breeding. Purdue Univ. Press, West Lafayette, IN.

Soost, R. K., and A. M. Torres. 1981. Leaf isozymes as genetic markers in citrus. Proc. Int. Soc. Citriculture 1:7-10.

Soost, R. K., T. E. Williams, and A. M. Torres. 1980. Identification of nucellar and zygotic seedlings of *Citrus* with leaf isozymes. HortScience 15:728-729.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Bio. 98:503-517.

Stuber, C. W. 1989. Comparative studies using RFLPs and isozymes as molecular markers for the study and analyses of multigenic traits in maize, pp 103-106. In: T. Helentjaris and B. Burr (eds.), Development and Application of Molecular Markers to Problems in Plant Breeding. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stushnoff, C. 1972. Breeding and selection methods for cold hardiness in deciduous fruit crops. HortScience 7:10-13.

Sutka, J., and O. Veisz. 1988. Reversal of dominance in a gene on chromosome 5A controlling frost resistance in wheat. Genome 30:313-317.

Tanksley, S. D. 1983a. Gene mapping, pp 109-138. In: S. D. Tanksley and T. J. Orton (eds.), Isozymes in Plant Genetics and Breeding, Part 1A. Elsevier, Amsterdam.

Tanksley, S. D. 1983b. Molecular markers in plant breeding. Pl. Mol. Biol. Rep. 1:3-8.

Tanksley, S. D., and J. Hewitt 1988. Use of molecular markers in breeding for soluble solids content in tomato--a re-examination. Theor. Appl. Genet. 75:811-823.

Tanksley, S. D., and T. J. Orton. 1983. Isozymes in Plant Genetics and Breeding, Parts 1A and 1B. Elsevier, Amsterdam.

Tanksley, S. D., and C. M. Rick. 1980. Isozymic gene linkage map of the tomato: applications in genetics and breeding. Theor. Appl. Genet. 57:161-170.

Tanksley, S. D., R. Bernatzky, N. L. Lapitan, and J. P. Prince. 1988. Conservation of gene repertoire but not gene order in pepper and tomato. Proc. Natl. Acad. Sci. USA 85:6419-6423.

Tanksley, S. D., H. Midina-Filho, and C. M. Rick. 1981. The effect of isozyme selection on metric characters in an interspecific backcross of tomato--basis of an early screening procedure. Theor. Appl. Genet. 60:291-296.

Tanksley, S. D., H. Medina-Filho, and C. M. Rick. 1982. Use of naturally occurring enzyme variation to detect and map genes controlling quantitative traits in an interspecific backcross of tomato. Heredity 49:11-25.

Tanksley, S. D., N. D. Young, A. H. Paterson, and M. W. Bonierbale. 1989. RFLP mapping in plant breeding: new tools for an old science. Biotechnology 7:257-264.

Thompson, J. N., and J. M. Thoday. 1979. Quantitative Genetic Variation. Academic Press, New York.

Torres, A. M. 1983. Fruit trees, pp. 401-421. In: S. D. Tanksley and T. J. Orton (eds.), Isozymes in Plant Genetics and Breeding, Part 1B. Elsevier, Amsterdam.

Torres, A. M. 1989. Isozyme analysis of tree fruits, pp 192-205. In: D. E. Soltis and P. S. Soltis (eds.), Isozymes in Plant Biology. Dioscorides Press, Portland, OR.

Torres, A. M., R. K. Soost, and U. Diedenhofen. 1978. Leaf isozymes as genetic markers in citrus. Am. J. Bot. 65:869-881.

Torres, A. M., R. K. Soost, and T. Mau-Lostovicka. 1982. Citrus isozymes: genetics and distinguishing nucellar from zygotic seedlings. J. Hered. 73:335-339.

Torres A. M., T. Mau-Lostovicka, T. E. Williams, and R. K. Soost. 1985. Segregation distortion and linkage of *Citrus* and *Poncirus* isozyme genes. J. Hered. 76:289-294.

Vallejos, C. E. 1983. Enzyme activity staining, pp 469-516. In: S. D. Tanksley and T. J. Orton (eds.), Isozymes in Plant Genetics and Breeding, Part 1A. Elsevier, Amsterdam.

Vallejos, C. E., and S. D. Tanksley. 1983. Segregation of isozyme markers and cold tolerance in an interspecific backcross of tomato. Theor. Appl. Genet. 66:241-247.

Vallejos C. E., S. D. Tanksley, and R. Bernatzky. 1986. Localization in the tomato genome of DNA restriction fragments containing sequences homologous to the rRNA (45S), the major chlorophyll a/b binding polypeptide and the ribulose bisphosphate carboxylase genes. Genet. 112:93-105.

Vardi, A., and E. Galun. 1988. Recent advances in protoplast culture of horticltural crops: *Citrus*. Scientia Horticulturae 37:217-230.

Vardi, A., and p. Spiegel-Roy. 1978. Citrus breeding, taxonomy and the species problem. Proc. Int. Soc. Citriculture 51-57.

Weeden, N. F. 1983. Evolution of plant isozymes, pp 175-204. In: S. D. Tanksley and T. J. Orton (eds.), Isozymes in Plant Genetics and Breeding, Part 1A. Elsevier, Amsterdam.

Weeden, N. F. 1989. Application of isozymes in plant breeding, pp 11-54. In: J. Janick (ed.), Plant Breeding Reviews, Vol 6. Timber Press, Portland, OR.

Weiser, C. J. 1970. Cold resistance and injury in woody plants. Science 169:1269-1278.

Wiltbank, W. J., and T. W. Oswalt. 1983. Laboratory determination of the killing temperature of citrus leaves during the 1981-82 and 1982-83 low temperature period. Proc. Fla. State Hort. Soc. 96:31-34.

Wiltbank, W. J., and T. W. Oswalt. 1984. Low temperature killing points of citrus leaves from spring 1983 to summer 1984. Proc. Fla. State Hort. Soc. 97:31-33.

Yelenosky, G. 1978a. Cold hardening 'Valencia' orange trees to tolerate -6.7°C without injury. J. Am. Soc. Hort. Sci. 103:449-452.

Yelenosky, G. 1978b. The effects of withholding water on cold hardiness of 'Valencia' orange and 'Star Ruby' grapefruit trees in controlled freezes. Proc. Fla. State Hort. Soc. 91:18-20.

Yelenosky, G. 1985. Cold hardiness in citrus. Hort. Rev. 7:201-238.

Yelenosky, G. 1990. Survival of young cold-hardened 'Hamlin' orange trees at -6.7C. HortScience 25:98-99.

Yelenosky, G., C. J. Hearn, and W. C. Cooper. 1968. Relative growth of trifoliate orange selections. Proc. Am. Soc. Hort. Sci. 93:205-209.

Young, N. D., D. Zamir, M. W. Ganal, and S. D. Tanksley. 1988. Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *TM-2a* gene in tomato. Genet. 120:579-589.

Young, R. 1963. Freeze injury to young seedlings of citrus cultivars and related species in the lower Rio Grande Valley. J. Rio Grande Valley Hort. Soc. 17:37-42.

Young, R. 1966. Freezing points and lethal temperatures of citrus leaves. Proc. Am. Soc. Hort. Sci. 88:272-279.

Young, R. 1969. Cold hardening in citrus seedlings as related to artificial hardening conditions. J. Am. Soc. Hort. Sci. 94:612-614.

Young, R. 1970. Induction of dormancy and cold hardiness in citrus. HortScience 5:411-413.

Young, R., and A. Peynado. 1967. Freezing and water-soading in citrus leaves. Proc. Am. Soc. Hort. Sci. 91:157-162.

Young, R., and C. J. Hearn. 1972. Screening citrus hybrids for cold hardiness. HortScience 7:14-18.

Young, R. H. 1961. Influence of day length, light intensity, and temperature on growth, dormancy, and cold-hardiness of Red Blush grapefruit trees. Proc. Am. Soc. Hort. Sci. 78:177-180.

Young, R. H., and A. Peynado. 1962. Growth and cold hardiness of citrus and related species when exposed to different night temperatures. Proc. Am. Soc. Hort. Sci. 81:238-243.

Zamir, D., and Y. Tadmor. 1986. Unequal segregation of nuclear genes in plants. Bot. Gaz. 147:355-358.

Zamir, D., T. Selilaben-David, J. Rudich, and J. A. Juvick. 1984. Frequency distribution and linkage relationships of 2-tridecadone in interspecific segregating generations of tomato. Euphytica 33:481-488.

BIOGRAPHICAL SKETCH

Richard Earl Durham was born June 27, 1961, in Danville, Kentucky. His primary and secondary education was obtained in the public school system of Lincoln County, Kentucky, where he graduated in May, 1979.

Mr. Durham attended the University of Kentucky from 1979 until 1983. He received a Bachelor of Science in Agriculture degree with a major in horticulture in August, 1983, graduating with distinction. During this time, he was employed as an undergraduate research assistant in the lab of Dr. Glenn Collins of the Agronomy Department.

In January, 1984, Mr. Durham was admitted to the Graduate School of the University of Florida. In August, 1986, he received the Master of Science degree with a major in horticultural science (fruit crops). His thesis research was directed by Dr. Gloria Moore and involved the characterization of isozymes as genetic markers in peach. Mr. Durham chose to continue in the program of Dr. Moore for his Ph.D., working in the area of citrus molecular genetics.

Following graduation, Mr. Durham will assume a postdoctoral position in the Agronomy Department at the University of Georgia. There he will be working on a project with Dr. Wayne Parrott to develop a genetic transformation system in peanut.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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